

22 January 1981

Towards what shining city, which hill?

The American presidency is an impossible job which has an enlivening effect on those who do it. New incumbents embark on tasks widely held to be beyond the resources of one man with the zeal and enthusiasm of an army. Both Presidents Kennedy and Johnson, when new to office, offered the promise of a fresh start on old problems, domestic and international. Given the persistent importance of the United States in the affairs of most other communities, the promise of a change of direction echoes around the world. Some of the consequences are unsettling; changes of direction are disconcerting. Some are also enlivening, as for the new incumbent himself. It is too soon to know what will be the consequences of Mr Reagan's accession to the presidency, but a single inauguration speech is not a sufficient guide to what will follow in the months ahead. The safest assumption is that Mr Reagan, like all his predecessors, will be good at some things and less good, or even frankly bad, at others. There is no shame in that, given that the job is everywhere acknowledged to be impossible. After a spell in which the president seemed to be bad — but not for want of trying to be good — at most things, it is no wonder that Mr Reagan's coming has cheered even his political opponents. For a time, at least, people will be prepared to give President Reagan the benefit of the doubt. The vacillations and exaggerations of the election campaign will be forgotten. To that rule, there will be only one exception. In his acceptance speech in Detroit last summer, Mr Ronald Reagan promised that he would lead the American people to a "shining city on a hill". That phrase, redolent of Blake or Bunyan, is too arresting to forget.

President Reagan, no doubt less innocent than he seems, will not be starved of advice, solicited and otherwise. Politics being what it is, his presidency may well be made or broken in the next few months by almost accidental issues — the inflation rate, or the problems of Central America. In these connections, no new president would choose to start from where President Reagan must. One of the perils of the job is that he has no choice. Another is that the issues, although recently made clear, are by no means irrelevant to what may happen on the wider stage. If inflation continues as it has been in the past few years, American institutions will be weakened as surely as they have been undermined elsewhere, then the foundations of the shining city will be found not to exist. Already many of the new occupants of offices in the White House must be regretting that there had to be a campaign, and a promise of a tax-cut to go with it. If the problems of democracy in Latin America are ignored or, worse, supposed to be familiar problems susceptible to old-fashioned solutions, the roof could fall in. Even President Reagan's friends will be well aware of the seriousness of these dangers. Those who heard his acceptance speech may be forgiven for looking further ahead.

The Reagan position, so often defined in the past several months that it has become unclear, is ambiguous on two crucial issues — arms control and academics. In the parochial spirit in

which trade magazines such as *Nature* grind their own axes on occasions such as the inauguration of a new president, special pleading may be excusable. On the face of things, for example, there is no reason why the new Administration should give an instant's thought to the special interest of the scientific community in the management of the arms race. Scientists, the argument might go, have (among other things) a special competence in military research and development. But only taxpayers have a right to say what should be done about relationships between the superpowers. President Reagan has in any case come a long way since the beginning of the election campaign, when he was bent on seeming a 1950s hawk. Now (the argument continues), he is for renegotiation of Salt II and other accommodations of that kind. The argument misses a point that will quickly become apparent, that the special but not exclusive interest of the scientific community in arms control is not merely academic. There is a sense in which J. Robert Oppenheimer was right in saying that "the physicists have known guilt" — hawks among them have persuaded doveish graduate students to work on unwelcome projects since time immemorial. The point has been reached at which both hawks and doves share with the general public the belief that it would be best if the consequences of the products of their joint endeavours could be contained within bounds, and that more or less any bounds would be better than none. This does not imply that all scientists share with the *Bulletin of the Atomic Scientists* the view that international tensions have worsened in the past few weeks — the *Bulletin* advanced its notorious minute hand one minute last week (and was mentioned on the British Broadcasting Corporation's morning news programme as a consequence) but did not convincingly explain why it had done so. Yet there is a great majority in the scientific community that would wish something sensible done about arms control, and which knows that such a course is not unattainable. The political question for the new president is whether he can see this as an opportunity.

Another issue for President Reagan, not too long to be left on the back burner, is that of the research community itself. Mr Carter (or, more probably, Dr Frank Press) has left the incumbent President with an awkward choice. Should an expansionary science budget be cut in the interests of financial prudence, or left as it is so as not to give offence? Neither of these questions should bother President Reagan in these early heady days. There is a simpler question he must answer. Since his early days as Governor of California, he has cut the figure of an adversary of the universities. At one stage, he forced Mr Clark Kerr out of his post as president of the University of California. Since then, he has made clear his disdain for academic preoccupations — decision is what matters. Such a position can be defended but is not defensible. The President needs urgently to build a bridge to the academics. To fail to do so will be to alienate some of those who might build a city, or find a hill.

Responsibility for trust in research

The public complaint, fashionable in the 1970s, that scientists could not be trusted when making statements about the hazards of pollution or of genetic manipulation, is now mercifully abating. Is the complaint now about to be replaced with the suspicion that scientists cannot trust each other? This will be some

people's first reaction to the article by Harris *et al.* on page 228, and our Washington Correspondent's commentary on it (page 227). The reaction is understandable, even forgivable. For what has emerged is that three out of four laboratory cultures of cells originally described in 1977 as derived from the spleens of patients



with Hodgkin's disease have nothing to do with human malignancy but are, instead, almost certainly derived from owl-monkey kidney tissue. This improbable conclusion has been established by means of as neat a piece of detective work as can be found in the recent literature. The fact remains that the four cell cultures have been the basis, in the past four years, for a spattering of spurious data in the scientific literature. Some of these unwarranted conclusions have been used by others in unrelated work towards the understanding of Hodgkin's disease. Elsewhere, laboratories have tried (usually without success) to establish their own analogous cell cultures from patients with the disease. But Dr John Long's apparent success with his supposedly authentic cultures appears to have led to the award of at least one substantial research grant (for \$500,000 over three years), afterwards rescinded. Especially because the principal investigator had been accused of falsifying data in a different context, many will now be tempted to suppose that four years of work with Hodgkin's disease has been a kind of hoax.

To jump to that conclusion would be wrong. Those who work with cells in culture are continually aware of the risks of contamination. Especially in laboratories specializing in this work, one established cell culture may be contaminated with cells from another by a variety of means — contaminated glassware, people's hands or defects of laboratory plumbing. Almost by definition, the most esoteric kinds of cells, requiring the most elaborate mixture of nutrients to make them grow, are those which are the most susceptible to contamination. HeLa cells, the malignant cells derived from a single tumour half a century ago, and widely used as models of malignant cells, are frequent contaminants. Inevitably, when a cell culture that grows only with difficulty is contaminated by cells that grow more vigorously, like HeLa cells, it is a matter only of time, perhaps days, before the original cells disappear, swamped by their more strongly growing competitors. Naturally, people with cells at risk are constantly on the look-out for contamination. This is obviously easiest when the cells at risk can be easily recognized, perhaps because of some distinctive biochemical characteristic. Again, however, and almost by definition, novel types of cells are likely to be least easily recognizable. In other words, it is entirely possible that people may work for weeks or months with cells from a tissue culture without knowing that the originals have been supplanted. It is less easy to see how meaningful results can be wrung from such a culture, but nothing is impossible. The fact that Harris *et al.* have been able to say no more about the fourth of the cell lines than that it is of human origin emphasizes the difficulties of identification. There is therefore no reason to doubt Dr Long's assertion (see page 227) that he believed the cell lines to be authentic; but his response to the doubts raised within and from outside his laboratory seems to have been too casual.

The significance of the paper by Harris *et al.*, for all its interest, should therefore not be exaggerated. Almost certainly, this is not the first occasion on which research reports based on the mistaken identity of tissue culture cells have appeared in the scientific literature. Most of the articles concerned have probably disappeared from people's citation lists as their data have been recognized as spurious or simply puzzling. It would be foolish to ask that the scientific enterprise should be carried on in such a way that such unwanted and unrecognized noise is entirely eliminated. Would intending authors be required to submit with their manuscripts samples of their tissue culture cells for validation by the referees? Would those working with supposedly pure and authentic strains of laboratory mice be expected to follow suit? And how would the scientific journals cope with the problems that would follow? The only reasonable conclusion is that the ideal is probably unattainable, that the scientific literature is based to a very large extent on trust in what its authors say, that the system works surprisingly well and that its occasional failures can be accommodated without too much difficulty. If it follows that the scientific literature is not to be regarded as a collection of Mosaic tablets, nobody will be the loser.

The problems created for laboratories by such developments as those identified by Harris *et al.* are more immediate and in some

ways more difficult. The necessarily close working relationship between people sharing the same facilities and even the same objectives in research is one of the most powerful safeguards of the authenticity of the scientific literature. However great may be the temptation for individuals to make too much of preliminary findings or to apply cosmetic treatments to otherwise untidy data, the knowledge that their most candid critics are likely to be their closest colleagues is constantly an influence towards sobriety. It is true, of course, that individuals who have misled themselves can usually succeed in misleading more junior colleagues as well, but even this is an unsure calculation now that many graduate students are zealous custodians of what is right and proper. The more certain safeguard, however, is that people in laboratories where all researchers are in the habit of talking freely with their immediate colleagues about their work, not merely at formal colloquia but over lunch, are imperceptibly persuaded to couch their interpretations of their work in moderate language. (The so-called sociologists of science, always on the look-out for subjects to study, could profitably pay some attention to the sociology of the laboratory as a formative influence on the pattern of science.) No doubt one of the reasons why the most successful laboratories are frequently large laboratories derives from the influence of these local invisible colleges.

Why, then, does this system of polite and imperceptible persuasion by colleagues not always function as it should? In the past few months, the grant-making system has frequently been blamed for departures from the path of strict propriety. The competition for grants, the argument goes, is now so fierce, and the consequences of failing to secure a grant can be so serious for the career of an individual, that people are tempted beyond endurance. This danger is more apparent than real. If the competition for grants is now more fierce, so is the scrutiny of the peer-review committees on whose recommendations most grants are awarded. A more likely explanation is that the grant-making system has disturbed the balance there would otherwise be among colleagues working within the same laboratory. A successful grant-holder tends, when grants are hard to come by, to acquire a status and a degree of autonomy that may not be entirely justified by his colleagues' estimate of his work. And people who are seen to enjoy the goodwill of the grant-making agencies are, if they so choose, to some extent immune from the necessarily searching scrutiny of their immediate colleagues. The Massachusetts General Hospital, one of the best known components of the Harvard medical complex, moved quickly after Dr Long's resignation last year to promise an audit of his work, and the paper by Harris *et al.* is one result. The hospital has probably for the time being done everything it can. But, in the long run, there and elsewhere, the objective should be to make sure that laboratory communities remain as rigorous as they should be in their internal assessment of all their members.

The responsibility lies fairly and squarely with heads of laboratories and heads of university departments. It is an unpalatable responsibility. But unless it is exercised diligently, the result will be unpalatable for the scientific community as a whole. One obvious danger is that of the emergence within the scientific profession of groups of zealous and often over-zealous people dedicated to rooting out what they consider to be dishonesty. Already there are signs of such developments. Some of the allegations of plagiarism in the past year or so appear to have had their roots as much in self-righteousness as in a concern for honesty in the literature. Some of the consequences of these allegations have been unfair to those accused. Yet there is no reason to suppose that the few cases of dishonesty that have come to light are in any sense the tip of an iceberg. On the contrary, as the day to day affairs of reputable scientific journals bear out, there is a general and quite remarkable concern for truth. Would-be authors are forever going back to their benches to check small points raised by referees, who in turn appear almost masochistically prepared to recommend the publication of data or arguments conflicting with their own positions. It would be tragic if these civilized habits were to be corrupted by the activities of self-appointed vigilantes.

More pressures on Rothschild system

Geology project threatened by more cuts

The Rothschild principle, whereby British government departments ("customers") commission research from research councils ("contractors"), already corrupted in medical and agricultural research, is now showing signs of strain elsewhere. The latest development is that two of the customers of the Natural Environment Research Council, the Department of the Environment and the Department of Industry, are threatening to cut the amount of geological research they are prepared to commission.

At stake are the Geological Survey and the mineral reconnaissance programme, both run by the council's Institute of Geological Sciences. The Department of the Environment, which currently contributes £1.5 million to the £4.5 million annual budget of the Geological Survey, is cutting its contribution to geological generally from £3.5 million in 1980-81 to £2.6 million in 1981-82. Although the department has not yet specified its priorities, it is expected that the cuts will be made in the Geological Survey and the engineering geology and bulk minerals resources programmes. The Department of Industry, which is the sole supporter of the mineral reconnaissance programme, is threatening to cut that budget from about £1.2 million in 1980-81 to £0.8 million in 1981-82.

Both departments say that they no longer want to fund research which is not directly relevant to their work. Thus the Department of the Environment will only fund those parts of the Geological Survey related to planning permission. Alternative funds for the survey are unlikely to be forthcoming and it will have to make do with less. Dr G. M. Brown, director of the Institute of Geological Sciences, finds his planning hampered by the Department of the Environment's delay in deciding its new priorities.

The Department of Industry is hoping that the mineral reconnaissance programme will find alternative funds from industry. Since 1972, this unit has been searching for metalliferous ores in Britain, especially in the Scottish Highlands, North Wales and Devon and Cornwall. Although deposits of several minerals — in particular copper and tungsten — have been found, only one, a barites deposit in Scotland, has led to an application for mining rights by private industry. Nevertheless, industry is interested. Companies consider that the

results provide useful information for assessing mining profitability in the light of future market trends. But hitherto, there have been no direct commissions from industry.

The Natural Environment Research Council has been trying to establish whether it could act a contractor for private industry. Meetings with the mining houses have thrown up several problems such as that of confidentiality (reports have previously been publicly available) and the assignment of mining rights. Industry itself is keen that the programme should

continue, but considers that financial support for it is properly the duty of central government.

Time is now running out. The new financial year begins in April, and twenty of the mineral reconnaissance staff have already moved to oil surveys of the North Sea, paid for by the Department of Energy. The Department of Industry has not finally decided to cut its support, but is thought by several observers to be in a mood to say that if private industry does not produce some cash, it will be a sign that the programme has little practical relevance. **Judy Redfearn**

Carter's last budget asks for more

Washington

Determined to enter the history books on an optimistic note, the outgoing Carter Administration has proposed to Congress a budget for the next fiscal year that contains a 4.3 per cent real growth in support for basic research.

"This is the best budget for the past four years for science and engineering research", said Dr Frank Press, the President's Science Advisor and director of the Office of Science and Technology, commenting on the budget proposals last week. He added that it confirmed President Carter's commitment to the support of science and technology "as an investment in the future".

In addition to the intended growth above the expected level of inflation, Dr Press singled out several initiatives that were being proposed by the outgoing Administration "to solve problems that we have known about for a number of years".

One of these is the proposed inclusion in the budget of the National Science Foundation of a \$75 million fund to improve university research equipment and laboratories. Their rapidly deteriorating state was highlighted in a recent report from the Association of American universities.

Two other proposals involve traineeships and other awards to avoid potential manpower shortages in fields such as computer science and energy engineering, and efforts to meet the present difficulties of engineering schools where, according to Dr Press, "faculty and equipment are not on a par with what one would expect in industry".

The big question, of course, is how much of this increase will escape the rapidly sharpening budget knife of the incoming Administration of President Ronald Reagan. For many social welfare programmes, the writing is already on the wall, but for science and technology the signals are mixed.

On the one hand, Mr Reagan's budget director, Mr David Stockman, has been talking about the need to rewrite the Carter budget "from top to bottom". Mr

Stockman has previously placed the National Aeronautics and Space Administration (NASA) among his "low priority" agencies which might absorb significant cuts. In the same vein, a group known as the National Tax Limitation Committee put out a report last week suggesting, for example, reduced spending on the Galileo mission to Jupiter — already being delayed a year by NASA because of delays with the launch vehicles — on its list of "expenditure control opportunities". The group is also raising questions about the appropriateness of government support for the space shuttle, and suggesting a re-examination of the future cost-benefit ratio.

At the same time, however, members of the various transition teams which have been established by the Reagan Administration to look at research and development programmes have been making optimistic noises, insisting that the new president is committed to the support of "science, technology and productivity".

Part of the increase in defence spending supported by both the Carter and the Reagan Administrations, for example, is likely to have beneficial spin-offs for research support, particularly in areas where the large aerospace companies have a stake. "I have been looking in teacups for the past two months, and each time I look I see a different picture", said outgoing NASA director Dr Robert Frosch last week.

The details of the Carter budget proposals contain several proposed new scientific starts. The NASA budget, for example, contains funding for the development of the Venus Orbiting Imaging Radar — announced by President Carter shortly before the election — as well as a new Geological Applications Program (GAP) which will use remote sensing satellites to study geological resources which might contribute to the discovery of new oil and gas deposits.

Reflecting the delays in the space shuttle programme, the proposed commitment to start work on a fifth shuttle orbiter, the

additional costs of science projects such as the space telescope, and the new projects described above, the total requested budget for NASA comes to \$6,700 million. This would be a 20 per cent increase over the budget for the fiscal year 1981 which began last October, and if it is allowed to stand, would be the largest annual increase in the agency's budget since the early 1960s.

The National Science Foundation has also put in for a hefty 23.5 per cent increase, from \$1,096 million in the current year to \$1,353.5 million next year. Most of this reflects the Carter Administration's keenness to support both research and training in engineering fields. The new engineering directorate will receive a 20 per cent increase in its research budgets.

At the National Institutes of Health (NIH), the proposed increase for biomedical research is less spectacular. On the basis that whatever the president asks for is traditionally increased by Congress, Mr Carter is suggesting that the NIH budget for basic research be raised by 9.4 per cent. Allowing for inflation, this would result in a drop of 1.1 per cent between 1981 and 1982.

At the Department of Energy, increased support for research into synthetic fuels and nuclear power — particularly fusion energy — has resulted in a requested increase of 9.4 per cent in real terms for basic research, second only to that of NASA.

Many of these figures will remain only as indications of the "good intentions" with which the Carter Administration is leaving office. Perhaps of more lasting significance are the figures prepared by Dr Press to demonstrate the main trends of federal support for science during Mr Carter's four years in the White House.

These reveal, for example, that overall the biggest winner as far as support for basic research is concerned has been the Department of Defense. If the proposed 1982 budget figures are taken into account, the Pentagon's basic research efforts will have grown by almost a quarter — 22.6 per cent — between 1978 and 1982.

Next come NIH. Congressional enthusiasm has raised the NIH research budget by 13.3 per cent over the four years, compared with a growth of 12.8 per cent at the Department of Energy.

The National Science Foundation, even if it is granted this year's large increase, will still only have seen its basic research grow by 9.2 per cent. And at NASA, reflecting the pressures which the space shuttle has imposed on the space science programmes, the basic research budget actually fell, in real terms, by 0.6 per cent over the same four years.

Overall, the growth in basic research, including the 1982 proposals, would come to 10.8 per cent for the period of the Carter Administration. In current dollars, the budget would grow by 58.2 per cent, from \$3,704 million to \$5,801 million.

Ironically, the research and development budget shows an identical increase of 58.2 per cent from \$26,388 million to \$41,734 million.

"Anyone who says that we do not engage in long-term planning is proved wrong by these figures", quipped Dr Press — expected soon to be elected president of the National Academy of Sciences — although he added that the agreement was actually fortuitous and that "the figures just happened to fall our this way". In practice, he will not be required to explain why it should be otherwise.

David Dickson

Argentinian power

Soviets help

Argentina has bought five tonnes of heavy water from the Soviet Union under International Atomic Energy Agency safeguards, the Argentinian Comisión Nacional de Energía Atómica announced last week. It is intended for "topping up" the Atucha-1 nuclear station, which needs on the average an annual heavy-water replacement of 1.5 tonnes.

The sale is part of the growth of Soviet-Argentinian trade since January 1980, when Argentina refused to back President Carter's embargo of grain sales to the Soviet Union. The Soviet Union is now Argentina's main market for agricultural products. In July of last year, Argentina's Secretary of Commerce, Alejandro Estrada, signed an agreement to supply the Soviet Union with 20 million tonnes of feed grain and soya beans during the next five years. There are also persistent rumours that a major agreement to export meat to the Soviet Union is now being negotiated.

In return, the Soviet side has shown considerable interest in Argentina's nuclear programme. Argentina has been conspicuous among third world countries since the early 1950s for its nuclear programme aimed at ultimate autonomy in both research and technology. The commission has announced that the target will be for practical purposes attained by the end of 1981, when the Córdoba uranium processing plant will begin producing an estimated annual production of 150 tonnes. Rafael Coppa, the director of the plant, said last year that Argentina will then have full control of the primary uranium cycle, from prospecting for

Promises for President Reagan to deny

Specific proposals included in the budget are as follows.

- Major difficulties with the development of a vehicle to launch the two Galileo spacecraft on their journey to Jupiter from the space shuttle have caused NASA to propose delaying the Galileo project for one year and switching to a new launch vehicle, a converted Centaur rocket.

- The Carter Administration proposes that the National Institutes of Health should aim to stabilize support for both competitive research grants and research traineeships. Last year, the Administration promised to provide enough money to keep the number of new and renewing competitive research grants constant at about 5,000. Given general fiscal constraints, however, this meant cutting back severely on the number of traineeships, a move which brought strong protests from various sectors of NIH, and was subsequently overturned by Congress.

- Defense Department support for research on US campuses seems destined to

continue to grow faster than support from any other federal agency. According to the budget request for the fiscal year 1982, military funding for research and development at US universities and colleges will be 21 per cent greater than in 1981, totalling \$639 million.

The proposed figure, most of which will be spent on unclassified basic research projects, is part of a 16 per cent increase in all military-sponsored basic research expenditure.

- One major new start proposed by the National Science Foundation (NSF) is the detailed design and initial construction of a 25-metre millimetre-wavelength radio-telescope which is planned for installation at Mauna Kea in Hawaii.

Plans for the new telescope have generated widespread support in the radioastronomy community. NSF has asked Congress for funds as part of a 29 per cent increase in the NSF budget for astronomical sciences, rising from \$58.5 million to \$75.6 million.

- The Carter Administration is proposing to cut support for research and development in the Environmental Protection Agency (EPA) from \$364 million to \$345 million. At the same time, the outgoing Administration wants to earmark an extra \$28 million to improve its review of the environmental impact of proposed major energy projects in the west of the country — particularly in connection with the synthetic fuels programme — and to launch a government-wide research programme on the effects of acid rain.

- The Carter Administration wants to give a major boost to research into magnetic fusion. The 1982 budget proposals include an increase of 28 per cent in fusion research, to a total of \$520 million in budget obligations. \$32.8 million of this would be spent on a new centre for magnetic fusion energy, as proposed by the Administration following a thorough review of the magnetic fusion programme last year. Research on magnetic confinement systems would increase from \$119 million to \$151 million.

David Dickson

uranium to putting the fuel elements into the reactor.

Nevertheless, the search for self-sufficiency does not rule out cooperative projects. Indeed, on returning from the World Energy Conference in Munich last September, Admiral Carlos Castro Madero, the president of the Argentinian commission, openly attacked the "negative aspects" of restrictions of nuclear technology transfer, adopted first by the "London Club" of nuclear suppliers and then (at the end of 1979) by a wider circle of industrialized Western states.

Soviet interest has grown in the past year. Last April, during a visit to Buenos Aires, the Soviet foreign trade vice-minister Aleksandr Manzhelo suggested a major nuclear cooperation between the two countries, stating at the same time that he thought that Soviet-Argentinian trade could well double in the next few years. At the end of July, Yuri Fokin, Secretary of the Soviet Foreign Ministry, visited the Atucha-I plant.

Vera Rich

Large electron project

Swedish cloud

Sweden is having second thoughts about participating in LEP, the 500-cm 500-GeV electron-positron colliding machine which, at a cost of 900 million Swiss francs, is planned to be the next major project of the European centre for high energy physics research, CERN. At a meeting of the Swedish Natural Sciences Research Council last month, 90 per cent of those present expressed doubts about the arrangements for funding the project.

Delegates from CERN's member states are expected formally to approve the building of LEP at the next meeting of CERN council in June. The plan is to finance the project out of CERN's annual budget by reducing expenditure on other programmes such as the intersecting storage rings and the synchrocyclotron. How quickly LEP can be built will depend on how much of the budget — SwFr610 million this year — can be diverted to it each year. What seems to be worrying Sweden is that the CERN council, which requires a two-thirds majority vote to approve budgets, could demand that Sweden pay more if the cost of LEP rises above initial estimates.

One faction of the Swedish research council says that LEP is simply too expensive to be built now. Another would agree to the project with some concessions — either that Sweden be made exempt from budget increases approved by CERN council, or that the CERN budget be divided into LEP and other programmes, giving Sweden the option of leaving LEP while remaining a full member of CERN's other activities. Under the present arrangement, a decision not to participate in LEP would effectively be a decision to

opt out of CERN altogether. An incidental factor which seems to have added weight to the arguments of LEP's Swedish opponents is the feeling that Swiss industry has reaped unfair benefit from contracts arising out of CERN's work.

Sweden's objections, which have come rather late in the negotiations (most of CERN's members have informally agreed to the LEP proposal), could delay official approval for the project. Although Sweden is not alone in wanting some guarantee that costs will not get out of hand, making it exempt from cost increases approved by CERN council is unlikely to be popular with other members: neither is splitting LEP from the rest of CERN's budget. Many see incorporating LEP into the annual budget as a way of controlling the rate at which money is spent on it.

At present Sweden contributes slightly less than 4.3 per cent of CERN's annual budget. If it decided to leave LEP, the other member states would have to decide how to redistribute the costs amongst themselves or whether to extend the time taken to build the machine. The chairman of the Natural Sciences Research Council, Mr Mats Lemner, expects that the council will shortly discuss the contribution with the Minister of Education. Depending on the outcome of this discussion, parliament may have to make the final decision. If it does, the Swedes would be able to meet CERN's June deadline for a decision on the contribution.

Judy Redfearn

Artificial hormones

European register?

Brussels

The European Commission is making heavy weather of its plan to ban the use of certain artificial hormones in animal farming. A meeting of agricultural ministers planned for last week was cancelled after the death of Mr F.O. Gundelach, the Danish agricultural commissioner. But the signs were that the meeting would have failed to reach an agreement.

The Commission decided last September that something should be done about hormones after the discovery that diethylstilboestrol was still being used for veal production in Italy. The hormone is banned in the United States and also in many European countries, but is so effective at increasing weight-gain in calves that, where its use is banned, black markets such as that in Belgium spring up.

The agricultural ministers were to have discussed two proposals elaborating on an original proposal made last December. The first calls for a register to keep track of all hormones used as medicinal products, whether for human or animal use, from manufacture and storage to distribution and final use. Veterinarians would be required to control all administration of hormones to animals. The proposed rules say that banned hormones can be used only for "therapeutic treatment" of

pathological cases diagnosed by veterinarians, and not for chronic use in preventive treatments. The second proposal is that there should be a comprehensive sampling system for testing animals and carcasses.

The proposals as drafted involve the "positive listing" of those hormones considered safe for use in meat production. This is opposed by Belgium and the United Kingdom. There is no dispute over banning compounds such as diethylstilboestrol, but it is held that a positive list would inhibit the development of new materials.

A system of "negative listing" would overcome some of these problems, but this would have to be continually revised as alternative hormones came on the market. Given the political need for action, the Commission favours the more cautious positive listing system. Whichever route is followed, the cost to European farmers of making the necessary adjustments will be substantial.

Jasper Becker

Soviet chemical industry

Effective economy

The Soviet chemical industry is rapidly acquiring prestige status in the Soviet media and ranks, according to a *Pravda* article last week, together with nuclear energy, space research and electronics, as one of the hallmarks of twentieth century progress. The proximate source of this accolade is not hard to identify: almost every article cites, at some point, Mr Brezhnev's dictum that "there can be no effective economy today without a modern large-scale chemical industry".

When Mr Brezhnev made this pronouncement at last October's plenum of the Central Committee of the CPSU, however, he was not so much commending the industry as calling for a programme of "resolute measures" for overcoming major shortfalls in chemical production, ranging from chemical fertilizers and plant protection agents to synthetic fibres, dyes and household detergents. The 33 per cent production increase specified in the guidelines for the new Five Year Plan for the chemical and petrochemical industries (recently placed under separate ministries) is, say the planners, essential if the shortfall is to be eliminated.

The chemical industry does not shoulder full responsibility for the gap. At the end of December, a *Pravda* editorial shifted at least part of the blame to other sectors. Fertilizer plants, said *Pravda*, were held up by insufficient supplies of natural gas and "inaccurate" planning by the light metallurgy sector. Plants with processes requiring high temperatures and pressures often cannot obtain corrosion-proof equipment. In some cases new factories have been built, without the necessary equipment being forthcoming, while, on other occasions, expensive installations have been purchased before it has been

finally decided just what the plant is to manufacture. The state supply board "Gosstab", *Pravda* concludes, must supervise the whole supply process more closely to eliminate such discrepancies.

Far more serious, however, are suggestions raised last week by a group of scientists from Byelorussia that the implementation of radical new technologies is being blocked by inter-departmental wrangles. In a major *Pravda* article they describe two such cases, in sectors which all planners consider to be of the highest priority — energy and agriculture. The first project, highly thought of by the Institute of High Temperatures of the Soviet Academy of Sciences, would have combined an electrolysis unit with a nuclear power plant so that hydrogen for fuel or industry could be produced during "off-peak" times. This combination of chemistry, electro-chemistry and nuclear power engineering ran into the stumbling block of what the group describe as the "excessively rigid specialization of the branches of industry".

The second project, for the production of protein biomass for animal feed using hydrogen-consuming bacteria, was worked out jointly by teams from the Byelorussian and Moldavian academies as long ago as 1976. To be cost effective, however, the byproducts of the synthesis — oxygen, nitrogen and possibly ammonia — would also have been exploited. All these are prime requisites of the mineral fertilizer industry, which itself produces waste gas with a usable hydrogen content. Yet although plans have been drawn up for a pilot biomass plant to be run in conjunction with the "Azot" fertilizer plant in Grodno, neither the Ministry of Chemical Fertilizer Production nor *Glavmikrobioprom* — the body in charge of microbiological production — can see its way to go ahead. **Vera Rich**

European research

Counting costs

Brussels

The knotty problem of putting a value on the European Community's research and development programmes is the subject of new proposals which the European Commission has just put forward. The objective of this soul-searching is to define criteria and methods for both evaluating and exploiting research. The idea stems not from a fit of self-doubt but from the Council of "Research" Ministers in December 1979. There are no hard and fast ideas in the document, but areas of investigation are instead defined. Four pilot projects have been approved, which will give independent experts free rein to put certain programmes under scrutiny. The commission proposes then to use the experience of the experts to formulate a

policy for exploiting and evaluating research results. This is to be ready by the end of the year but, before then, the first outlines of the policy will be presented at the International Congress for Research Evaluation in October.

One pilot project has already been completed by six independent experts, who looked into the Community's energy conservation and solar energy activities. Four other expert groups are to investigate programmes of research and development in fusion, radioactive waste management, the "Reference Bureau Programme" concerned with physical standards and critical data, and geothermal energy, the use of hydrogen as a fuel and work on the systems analysis of energy use.

The tightening of the European Community's budgetary belt is one motive for the new initiative, but another is the fear that the results of research are being ineffectively disseminated. The experts will be concentrating on the diffusion of information and in particular how to make the results available to the layman, requiring that more outlets should be found and language barriers overcome. Indeed, one member of the European Parliament has asked in a written question why the Commission does not publish the results of research in English as well as in the author's mother tongue. But the commission says that unless the results of research are first digested, readership is always restricted to specialist circles.

The stickiest part of the process on which the Community has embarked is the evaluation of research whose impact is likely to be long term or even unforeseen. Simplifying the results may help. Thus the report on solar energy and conservation says that the success of the programme cannot be measured in "tonnes of oil saved" and that the use made of research carried out may require further action and will take time. Even so, the chairman of the evaluation team, Ugo Forinelli, is confident that European research in this area is already competitive with that in the United States even though the European budget is much smaller than the American. Forinelli's team has made a number of recommendations about the management of the Community's programme, including the need for more explicit statements of objectives when putting contracts out to tender and some suggestions of areas where research might be concentrated or even discontinued.

The commission seems modestly aware of the inherent limitations of plans to evaluate research. The underlying objective is to strengthen the link between research and development and industrial innovation, and the commission acknowledges that its own research cannot be an important source of commercial inventions. But restrictions on Community expenditure outside the agricultural field are likely increasingly to stimulate the justifying of research. **Jasper Becker**

Swedish research

Lucky science

Stockholm

Research is one of the very few areas to be given more money in the budget bill for the fiscal year 1981–82, presented this week in Stockholm. Against the background of a budget deficit amounting to about \$15,000 million, cutbacks in social services and mounting economic gloom, research received an extra \$28 million.

Considering that this money is to be divided between the projects of eight ministries, individual increases will not be very great, and in real terms may well be eaten up by inflation, which was 14 per cent in 1980. One staff member at the Natural Sciences Research Council estimated that the net results of the council's \$2 million increase will be an unchanged level of activity. But this is better than a cutback, the fate of nearly all other sectors.

The government sees research strengthening Sweden's industrial competitiveness in the long term. The Minister of Industry, Nils Aasling, wants to develop sophisticated chemical and mechanical products, and is to make specific allocations for this purpose in the spring. One sign of this policy is the changing proportions of the space budget being allocated between international and national programmes. In the 1980–81 fiscal year, the Swedish Space Corporation's budget of about \$44 million was equally divided between international and national activities, but in future the accent will be on national programmes. The corporation's budget has been increased overall to about \$58 million — a real increase in spite of inflation — about half of this will be used to build the country's first space satellite, Viking, which is to be launched from the European rocket Ariane in May 1984. It is hoped to produce the Viking at half the cost of comparable satellites from other European countries, and the project is planned to give Sweden a profitable national space industry. Another project being planned is an experimental telecommunications satellite.

The lion's share of the increase in research expenditure — about \$16 million — is to be disbursed by the Education Minister, Jan-Erik Wickstroem, who wants to increase research capacity across the board. He is proposing an increase of about 12 per cent for mathematics and natural science faculties, an average of about 9 per cent for the four research councils as well as 90 new PhD places to be spread over all subjects, \$2 million for particularly expensive equipment, \$2 million to subsidize lecturers who want to take time off for research and \$1 million for research libraries. Fifteen new chairs will be set up, among them molecular genetics (Karolinska Institute), medicine (Uppsala) and astrophysics (Gothenburg).

Wendy Barnaby

Graduate education

Too many laggards

The Science Research Council, the chief source of public support for British graduate students, is alarmed at the lengthening time taken to complete PhD courses. A preliminary survey of 25 higher education institutions has shown that on average only 60 per cent of those holding SRC studentships complete their PhDs within four years. According to Sir Geoffrey Allen, chairman of the council, a figure of 80 per cent would be respectable, but 90 per cent would be the ideal.

The issue has come to the surface after an investigation by a working party of the Advisory Board for the Research Councils, which has been looking into the broader question of postgraduate education and manpower needs. That in turn was stimulated by the revelation of Sir Michael Posner, chairman of the Social Science Research Council, to the Public Accounts Committee of the House of Commons last summer that fewer than 30 per cent of his council's graduate students complete their PhDs within four years.

Given pause by that statistic, the Science Research Council made a rapid survey of five institutions where it supports students and arrived at the figure of 60 per cent, which a subsequent survey of 25 institutions has upheld. The advisory board's working party, which is also concerned with the performance of postgraduates supported by the Social Science, Medical and Agricultural Research Councils, has commissioned a more detailed study intended to throw some light on why so many students take so long to complete their theses, or even fail to complete them at all.

Particular attention is likely to be paid to the performance of the Science Research Council's students, if only because there are more of them than of the other councils — on the average, 2,350 new science studentships are awarded each year. Most of these are in the gift of university departments, to which studentships are allocated on a quota basis. Studentships are worth about £3,500 a year, and are tenable for three years, the estimated time for completing a research project.

The reasons for these delays are still obscure. The Science Research Council expects to find marked differences of performance in different institutions and subject areas. PhDs in pure science may more often be completed than those in applied science — applied scientists and engineers are more likely to find jobs in industry, where writing a thesis may seem irrelevant and where there is little time for writing up anyway. It is also suspected that institutions and departments with a large number of PhD students will have a better track record than those with relatively few.

The issue also, however, raises questions concerning the meaning and purpose of a

PhD, which the advisory board's working party is looking into under its broader remit. Should a PhD for example, be a thorough and lengthy investigation of a detailed scientific problem, or more simply a means of training a student in the techniques of research? The approach is bound to have implications for the completion time.

As yet, no reliable pecking order of institutions has been established, but the early surveys do suggest that the Universities of Birmingham, Cambridge, East Anglia and Bristol, and King's College in the University of London, have the best completion records and that the Universities of Newcastle upon Tyne, Sussex and Bradford, together with Imperial College, London, and most of the polytechnics, have the worst.

The poor track record of Imperial College, regarded as a highly prestigious scientific and technological institution, may seem surprising. Lord Flowers, rector of the college, says that the explanation may be that large numbers of its postgraduates rapidly find employment in industry, leaving them little time for writing up.

The advisory board's working party has yet to decide what should be done. Sanctions against departments with poor track records have been mentioned. Cutting quotas of studentships is an obvious device. Sir Geoffrey Allen, however, hopes to avoid such heavy-handed treatment. Most academics, he says, are willing to accept genuine criticism and put their houses in order. The peer review system should take care of that.

Judy Redfearn

British universities

More confusion

Confusion among British universities about their financial prospects appears to have been further deepened by the letter from the chairman of the University Grants Committee, Dr E. S. Parkes, circulated to vice-chancellors on 30 December. The letter contained a warning that the resources available for the 1981–82 academic year may be reduced by between 5½ and 6 per cent compared with the amounts advertised in the Public Expenditure estimates a year ago. The committee's latest estimate of the shortfall next year is an amalgam of a 3½ per cent cut estimated to be the universities' share of the £30 million cut for higher education announced last November and the still incalculable effect on university finances of the partial disappearance of overseas students, some of whom have been frightened away by "economic" fees.

Some universities regard Dr Parkes's warning as a signal for drastic belt-tightening. Last week, for example, Lord Annan, Vice-Chancellor of the University of London, told the university senate that

the total budget of £200 million might be reduced by between £15 and £20 million in 1982. Other universities appear to be taking a more phlegmatic line, believing that they cannot know the worst until the individual allocations of funds for 1981–82 are made in April or soon thereafter.

Between now and then, the committee itself will have several difficult questions to decide. One possibility raised in the letter from Dr Parkes is that the committee may keep back until later in the academic year a proportion of the funds made available by the Department of Education and Science, using the reserve to make good deficiencies that have by then appeared. There are precedents for such a reserve, but no decision has yet been made, nor have criteria for deciding how to use the money been defined.

The Parkes letter also promises explicit guidance in the spring on the numbers of home students at which British universities should aim in the coming academic year, and on their desired distribution among different kinds of courses. Although such "guidance" has accompanied previous financial allocations to British universities, some universities now apparently fear that the committee intends to be more "dirigiste" than in the past — while others remark that Dr Parkes's letter is a good deal less so than his speech to a closed meeting of the Committee of Vice-Chancellors at the beginning of December. It does however seem clear that the University Grants Committee will take steps to ensure that universities do not earn their way out of trouble by recruiting more home students than at present for the sake of the extra fee income that would bring.

Planning for the year ahead has been further confused by reports that Mr Mark Carlisle, Secretary of State for Education and Science, told a conference in the north of England on 6 January that he saw no reason why the cuts now proposed should be matched by "a reduced provision". Universities, on the other hand, say that there will have to be a reduction of student entry this October if they are to live within their straitened budgets.

The future constitution of the University Grants Committee itself also appears to be in question. Taking its cue from a recommendation of the House of Commons Select Committee on Education towards the end of last year that the committee should cultivate more independence, the department has raised the possibility that the committee's staff (at present seconded from the Civil Service) should become direct employees. Some of those concerned wryly reflect that this is not quite the independence that the House of Commons committee had in mind, but they acknowledge that such a step would reduce the size of the Civil Service and transfer part of its cost away from central government. The fine print in the proposal, which is said to be "very small", is being read carefully.

CORRESPONDENCE

Badgers guilty

SIR — In his letter published on 11 December 1980 (page 532), Stephen Harris, among many other misleading and biased remarks, stated that the rate of decline in the incidence of tuberculosis (TB) in badgers in the South West was "paralleled" by a decline in the incidence of reactors in cattle herds not only in the South West but also in the rest of England. This statement was based on a graph which was attached to a copy of the letter sent to Lord Zuckerman and which is now reproduced (Fig.1). It was deduced from this that there might well be some common cause unconnected with the gassing campaign that was reducing the incidence of TB both in cattle and badgers throughout the country.

This "parallelism", however, was only secured by expressing all plotted values as percentages of the corresponding 1974 values. In Fig.2 I have plotted the breakdowns for the South West and for the rest of England on the same scale, subdividing the South West into (a) Gloucester, Avon and Wiltshire; (b) Cornwall; and (c) Devon, Dorset and Somerset. The radical differences between the counties that have a high incidence of herd breakdowns, (a) and (b), and the rest of England (d) is immediately apparent.

Table 10 of the report (*Badgers, Cattle and Tuberculosis*, Lord Zuckerman; HMSO, London, 1980) shows that the sources of infection are indeed different in the South West and in the rest of England. In groups (a)

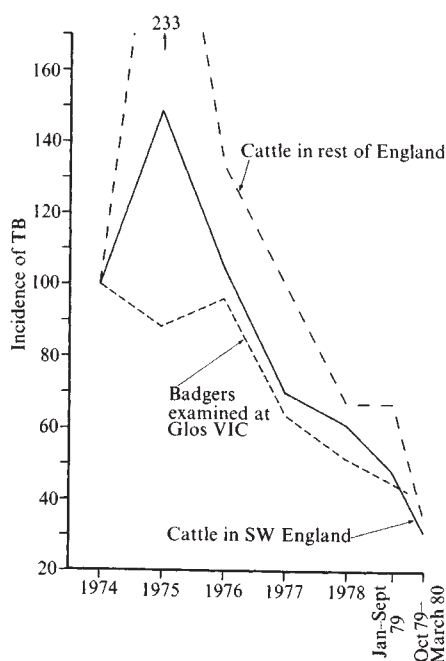


Fig.1 Annual changes in the incidence of TB in badgers sampled at the Gloucester Veterinary Investigation Centre (page 62 of the report) and herds of cattle in South-West England and herds of cattle in the rest of England (page 64). The first year for which data were available in the report from all three samples (1974) was taken as 100 per cent; for subsequent years, the level of TB was expressed as a percentage of the incidence in 1974.

and (c) three-quarters of the infections are attributed to badgers, but only 1 per cent to imported Irish cattle, whereas in the rest of Great Britain one half are attributed to imported Irish cattle and none to badgers.

In Cornwall (b), as Harris observes, only 15 per cent were definitely attributed to badgers, but as almost three-quarters of the causes of Cornish breakdown were recorded as "unknown" and the remainder were either from purchased cattle or contiguous premises, it may be presumed that infection from badgers was much greater than this.

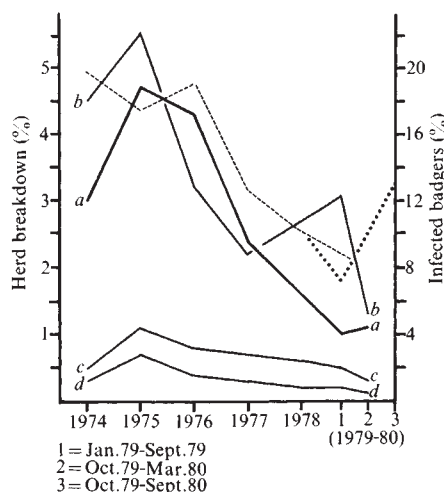


Fig.2 The percentages of herd breakdown are shown by full lines (from Table 19 of the report). The percentages of badgers found to be infected are shown by the broken line (Table 15). The dotted line gives some supplementary information from Table 18 (updated).

The fall in the incidence of herd breakdowns following the introduction of the gassing campaign in Gloucester/Avon agrees well with the reduction in the incidence of TB in badgers (broken line), bearing in mind that in addition to the reduction in the percentage incidence in badgers the numbers of badgers in close contact with herds must have been substantially reduced by the gassing.

The dotted line is derived from the latter part of Table 18, updated to September 1980, and indicates the rise in TB incidence in badgers following the cessation of gassing. (Updated figures for herd breakdowns in Gloucester/Avon and Wiltshire are not yet available).

The only support that Fig.2 gives to the suggestion that there was a common cause, other than appropriate action by ministry officials, affecting the decline in TB operating throughout the country is the substantial rise between 1974 and 1975. This cannot be related to infection from badgers. It may possibly have been due to climatic factors or to some improvement in the tuberculin test.

Figure 1 is a sad illustration of the way in which distorted and partial graphical presentation can be used for propaganda purposes.

FRANK YATES

Rothamsted Experimental Station,
Harpenden, UK

Rothschild retreat

SIR — It is not clear from your leading article in the January 1/8 issue (page 2) whether you regret the government's "retreat from Rothschild" or just the manner in which it is happening.

One of the difficulties for the Medical Research Council (MRC) at the time of the Rothschild report was the uncertainty as to the failures in the then current system which it was intended to correct. The Ministry of Health did not identify any omissions on the part of the MRC in their contribution to improving the health of the nation, nor did they subsequently propose contracts in fields of work outside the previous commitment of the MRC. Rothschild, in a thoughtless comment, referred to the very low expenditure on research into ageing, one of the fundamental aspects of all living things, but the understanding of which is nowhere in sight. He failed to ask the practical question as to how much of the MRC budget was spent on investigating diseases most common in old age. The answer would have been a major part.

There are, at present, three developments in medical research on which great hopes of practical gain are based: interferon, antibody production by hybridomas and DNA technology. How far the expectations will be justified remains to be seen, but the heavy investments of pharmaceutical companies throughout the world in these fields is evidence that the hopes are held widely. MRC laboratories were responsible for the first two discoveries and made a very big contribution to the third. None came from planned research but from "Dr Gowans' doctrine of investment in good ideas and good people".

It has been argued strongly that much more attention should be given to public health and preventive medicine and that the MRC's expenditure has the wrong emphasis. But one of the major advances in this field, the correlation of the incidence of lung cancer with cigarette smoking, came from work in an MRC unit.

No doubt governmental administration of civil science could be improved substantially, but the next enquiry which you appear to be advocating must surely start by identifying the omissions and failures of the present system whether in medical, agricultural or any other field of research before recommending changes to overcome them. Your generalized denigration of a system which has achieved obvious successes is not enough.

R.R. PORTER

Department of Biochemistry,
University of Oxford, Oxford, UK

Creating problems

SIR — Your readers should know of the court action by creationists against the State of California, now expected to begin on 2 March. The suit, which is sponsored by the Creation Research Society of San Diego, is in the names of Segraves *et al.*, students in California schools, including children of Mrs Nell Segraves of the society. The suit charges that the State of California has violated the

Continued on page 335

NEWS AND VIEWS

Deep-ocean hydrothermal vent communities

from J.T. Enright, W.A. Newman, R.R. Hessler & J.A. McGowan

THE theory of plate tectonics has led to a completely unexpected bonanza of research problems for marine biologists. The theory predicted major heat input to the ocean in areas where the sea floor is spreading, and in the search for those sources, geophysicists discovered not only deep-ocean hydrothermal vents, where warm water emerges from subterranean sources, but also extraordinary concentrations of large and unusual animals surrounding the vents^{1,2}. The bottom of the deep sea typically has a sparse fauna dominated by tiny worms and crustaceans, with an even sparser distribution of macrofauna, including clams, crabs and echinoderms. Near the hydrothermal vents, however, are bizarre ensembles of abundant large animals — communities which resemble nothing ever before encountered by marine biologists. The most conspicuous and unusual animals around the warm-water vents ($\sim 20^\circ\text{C}$) are huge gutless tube-worms (Vestimentifera), some of which are over 3m long and up to 10 cm in circumference; but there are also remarkable densities of huge clams (25cm length is typical), blind crabs, fish and polychaetes (see figure). More than 100 species have been collected², many of which are elsewhere unknown.

How do the animals in these deep-sea communities find enough to eat? Elsewhere, the deep benthic fauna relies on particulate matter, ultimately derived from photosynthesis, falling from above. The food supplies necessary to sustain the vent communities, however, must be many times that ordinary fallout. In the first report which described the vent fauna, Lonsdale¹ proposed two possible sources of nutrition: advection of food materials from surrounding regions and bacterial chemosynthesis. Shortly thereafter three kinds of evidence were presented³ which support the idea of intense local chemosynthesis: (1) hydrogen sulphide, as a potential basis for fixation of CO_2 , was found in vent water; (2) some of the bacteria isolated from a vent site were found to be capable of chemosynthesis on a thiosulphate medium; and (3) bacterial

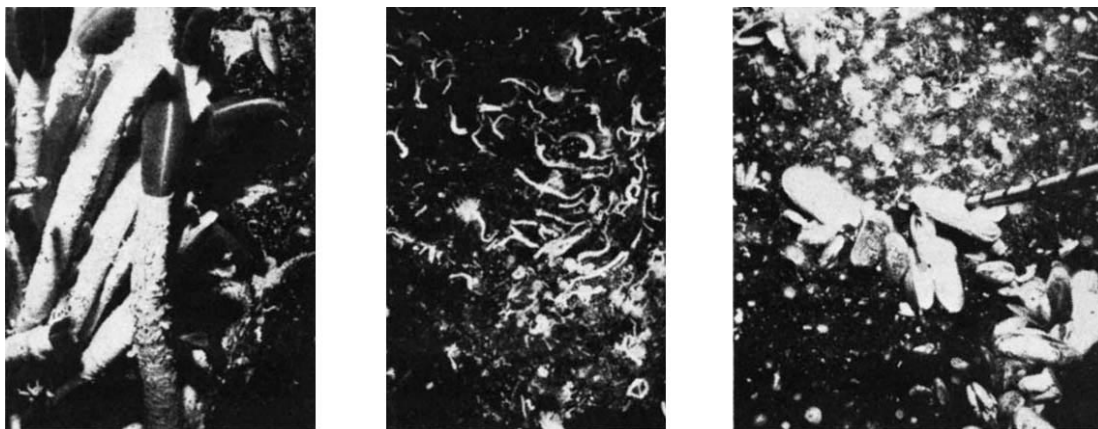
concentrations of 10^8 to 10^9 cells per ml were found in samples thought to be pure vent water. This final observation seems decisive: if such astonishing concentrations of bacteria — difficult to achieve even under optimal laboratory conditions (D.M. Karl, personal communication) — are indeed typical of steady-state vent outflow, then food from within the vent would be dwarfed by any contribution from advection. Hence, the widely quoted conclusion was reached, that bacterial chemosynthesis provides the foundation for hydrothermal-vent food chains²⁻⁵ — an exciting prospect because nowhere else on Earth is a major community of diverse organisms known which does not depend, at least indirectly, on photosynthesis.

There are, however, certain difficulties with this interpretation. Some of the large sedentary organisms associated with vents are found at ordinary deep-sea temperatures many metres from the nearest hydrothermal sources⁶, and therefore would not directly encounter any bacteria from within the vent. It is conceivable, however, that bacterial aggregates, which have grown subterraneanly, in the rising plume of vent water might rain back out in the peripheral areas to nourish these filter-feeders. Another difficulty is that similarly dense populations of large animals have been found^{2,7} in the proximity of 'smokers' — hot-water vents where the dominant, conspicuous flow emerges at temperatures up to 350°C . No bacteria can be expected to multiply or even survive such heat, and none was found in the hot vent water⁷. Unless the smokers are consistently associated with smaller but more hospitable warm-water vents² — a distribution that has not actually been observed (F.N. Spiess, personal communication) — the chemosynthesis hypothesis could at best account for only a fraction of the vent faunas.

The food resource question has been further complicated by a recent and more extensive investigation⁵ of the microorganisms associated with one of the warm-water vents in the Galapagos Rift

region. Bacterial concentrations were measured in near-vent water which were approximately three orders of magnitude lower than those initially reported (5×10^5 – 10^6 cells per ml). These values were confirmed by replicate estimates of biomass based on ATP concentrations in the water⁵ (100–250 μg of microbial carbon per litre, compared with the previous estimate³ of 100–1,000 mg of bacteria per litre). Although the authors of that report still endorsed the chemosynthesis hypothesis⁵, the available data suggest that Lonsdale's first alternative¹ deserves more attention than it has received. Could advection conceivably supply the near-vent fauna with enough food organic matter ultimately derived from photosynthesis?

Because of its temperature, water from a vent is very buoyant, densities as low as 0.62 gm cm^{-3} having been reported for a 'black smoker'⁸. The rising plumes of vent water are, however, rapidly diluted. Spiess *et al.*⁷ report that a vent discharging at 23°C into water at 1.8°C produced a temperature only 0.3°C warmer than ambient at a height of 10 m above the vent. These values indicate that within 10 m of the bottom, the advected diluting water was about 70-fold the discharge volume $[(\Delta T_1 - \Delta T_2)/\Delta T_2]$. Stability considerations dictate that such advective flow into the vent area would originate near the water-substratum interface, where suspended organic matter accumulates and epibenthic animals are concentrated. Typical values for suspended particulate matter in deep-sea waters within a few metres of the bottom^{9,10} are of the order of 50–500 mg m^{-3} , of which several per cent is organic matter. Taking a conservative estimate of 5 mg of particulate organic carbon per cubic metre and the calculated dilution factor of 70, we find that for every cubic metre of vent discharge, 350 mg of particulate organic carbon would be advected into the vent area. If discharge volume were to be, say, $1 \text{ m}^3 \text{ s}^{-1}$, advection could provide more than 30 kg per day of potential food. In addition, there is a good chance that small live animals in the advected water might be killed or stunned by thermal and/or



Examples of hydrothermal-vent fauna from Galápagos Rift area. Left, vestimentiferan worms, about 3 cm in diameter. Middle, dense group of encrusting serpulid polychaetes, about 5 cm long, some with tentacular crown extended. Right, vesicomys (white) and mytilid (grayish) bivalves adjacent to an aggregation of anemones. The tip of the probe is 1 cm in diameter.

chemical shock, and subsequently rain down, thereby contributing to the vent food supply. Hence, it appears that an input of non-photosynthetic food might not, at least in principle, be required by the vent animals.

Such input calculations, however, ignore what may be the most important aspect of advective processes, that the near-vent area should accumulate and concentrate most advected organic matter. Entrained food particles which are not consumed in their initial passage through the vent area would be wafted upwards, but ought thereafter (given a density greater than ambient seawater) to rain out predominantly on the near-vent substratum. Once on the bottom, this food material would not be lost to the vent ecosystem because of resuspension. Sediment-trap data¹¹ indicate that the particulates suspended in near-bottom water of the deep sea must, on the average, be resuspended every few days. Because of such resuspension, the initially advected material would be subject to repetitive re-entrainment, thereby enriching the advected waters. Hence, filter-feeding animals near a vent should experience not only increased flow rate of waters bearing organic nutrients, as suggested by Lonsdale¹, but also experience unusually high concen-

trations of suspended particulates from non-vent sources, food which is ultimately derived from sea-surface photosynthesis. The nutrition of filter-feeding animals is even more dependent on food concentrations than on the flow rate of surrounding waters. At any new vent site, even if chemosynthesis were to be negligible, this sort of accumulation should progress until catabolic degradation, by both micro- and macroorganisms, balances the net rate of advective input.

Both Lonsdale's suggestion that food materials would be advected from extensive areas surrounding deep-water hydrothermal vents, and the enhancement of the local density of these food materials due to gravitational fallout and recirculation, appear to be reasonable, if not logically inescapable, expectations. The as yet unresolved issue is the quantitative one — what proportion of the food supply for macroorganisms is provided by such advective input and what proportion by vent-related chemosynthesis? The suggestion that advective input alone might provide an adequate nutritional basis for near-vent food chains implies that animals relying on advected biomass ought to do well around any intense persistent deep-water source of heat — an idea which may be worthy of direct experimental test.

By similar reasoning, the near-vent area should also represent a zone of accumulation for any organic materials which might be produced by chemosynthesis, and therefore it may prove to be extremely difficult to distinguish between a predominantly photosynthetic and a predominantly chemosynthetic basis for the near-vent food chains by measuring the local density of organic matter and associated bacteria, or by examining the spatial gradients in those densities. Even samples thought to be pure vent water³ are not easy to interpret; the possibility of dilution or contamination by materials of non-vent origin will remain troublesome, because of the intense mixing around the vent⁵.

Some of these uncertainties and paradoxes may soon be resolved by carbon-isotope data. The stable isotope ratio, ^{13}C to ^{12}C , depends on the nature of an organism's carbon sources, and is also a sensitive indicator of alternative metabolic pathways involved in photosynthesis and of heterotrophic fractionation. In the tissues of some of the vent fauna, the $^{13}\text{C}/^{12}\text{C}$ ratio differs significantly from values typical for other deep-sea animals¹²⁻¹⁴, suggesting that some sort of unusual carbon sources or metabolic processes — perhaps chemosynthesis — may be important in near-vent areas. The finding that the ^{13}C ratios differ between vent bivalves and vestimentiferan worms in the direction of this anomaly (H. Craig, personal communication) is so far unexplained, the one group having smaller and the other larger values than expected. Also of great interest are data on the ratios of ^{14}C to ^{12}C in the tissues of near-vent animals, which ought to reflect their food resources, and which indicate¹⁴ an apparent age of about 2,500 yr. Intra-vent chemosynthesis would incorporate the dissolved inorganic carbon of vent water, 80% or more of which apparently comes from magmatic sources (H. Craig, personal communication). If one assumes that magmatic CO_2 is very old and contains negligible amounts of ^{14}C , then in-vent chemosynthesis should lead to ^{14}C ages ($>15,000$ yr) very different from those obtained for the tissues of vent macrofauna ($\sim 2,500$ yr). So far, however, no actual measurements of ^{14}C in vent water are available, and other interpretations about its probable ^{14}C age have been proposed¹⁴. The apparent age of 2,500 yr of the circumvent fauna is clearly much older than concurrent sea-surface photosynthetic production (expected age ~ 0), and much younger than the available data for organic carbon incorporated into the top few millimetres of deep-ocean

The authors are professors at the Scripps Institution of Oceanography, La Jolla, California.

1. Lonsdale, P. *Deep-Sea Res.* **24**, 857 (1977).
2. Ballard, R.D. & Grassle, J.F. *Natn. Geogr.* **156**, 689 (1979).
3. Corliss, J.B. *et al. Science* **203**, 1073 (1979).
4. Galápagos Biology Expedition Participants *Oceanus* **22** no. 2, 2 (1979).
5. Karl, D.M., Wirsén, C.O. & Jannash, H.W. *Science* **207**, 1345 (1980).
6. Killingley, J.S., Berger, W.H., MacDonald, K.C. & Newman, W.A. *Nature* **287**, 218 (1980).
7. Spiess, F.N. *et al. Science* **207**, 1421 (1980).
8. MacDonald, K.C., Becker, K., Spiess, F.N. & Ballard, R. *Earth planet. Sci. Lett.* (in the press).
9. Eitrem, S.L. & Ewing, M. in *Studies in Physical Oceanography Vol. 2* (ed. Gordon, A.L.) 123 (Gordon & Breach, London, 1972).
10. Jacobs, M.B., Thorndike, E.M. & Ewing, M. *Mar. Geol.* **14**, 117 (1973).
11. Gardner, W.D. thesis, Massachusetts Inst. Technol. (1977).
12. Rau, G.H. & Hedges, J.I. *Science* **203**, 648 (1979).
13. Rau, G.H. *Nature* (in the press).
14. Williams, P.M., Smith, K.L., Druffel, E.M. & Linick, T.W. *Nature* (in preparation).
15. Williams, P.M., Stenhouse, M.C., Druffel, E.M. & Koide, M. *Nature* **276**, 698 (1978).

A new stingray from South Africa

from Alwyne Wheeler

ICHTHYOLOGISTS are accustomed to the regular description of previously unrecognized species of fishes, which if not a daily event at least happens so frequently as not to cause great comment. Previously undescribed genera are likewise not infrequently published, but higher categories are increasingly less common. The discovery of a new stingray, which is so different from all known rays as to require both a new family and a new suborder to accommodate its distinctive characters, is therefore a remarkable event.

A recent paper by P.C. Heemstra and M.M. Smith (*Ichthyological Bulletin of the J.L.B. Smith Institute of Ichthyology* 43, 1; 1980) describes this most striking ray as *Hexatrygon bickelli* and discusses its differences from other batoid fishes. Surprisingly, this remarkable fish was not the result of some organized deep-sea fishing programme, but was found lying on the beach at Port Elizabeth. It was fresh but had suffered some loss of skin by sand abrasion on the beach, and the margins of its fins appeared desiccated in places. The way it was discovered leaves a tantalising question as to its normal habitat, but Heemstra and Smith suggest that it may live in moderately deep water of 400–1,000 m. This suggestion is supported by its general appearance (small eyes, thin black dorsal skin, flacid snout) and the chemistry of its liver-oil.

The classification of *Hexatrygon* presents some problems, the most obvious of which is that it has six pairs of gill openings, whereas all other batoid fishes (sawfishes, rays, skates, stingrays and electric rays) have only five paired gill openings. Another striking feature is that the spiracular openings behind the eyes are closed dorsally by a cartilage-supported flap to form an oblique slit. It seems that *Hexatrygon* may be able to shut its spiracles down externally, something that other batoids cannot do. Internal features are as strikingly different from its relatives, and the shape and structure of the snout are also unique. The snout is elongate, of uniform thickness (16–18 mm deep in the fish of 103 cm total length) and flaccid. Internally it is supported by lightly calcified rostral cartilages and filled with

an acellular jelly, while the underside is richly supplied with well developed ampullae of Lorenzini. These ampullae are known to be electroreceptors and assist in finding buried food organisms in shallow-water rays. The combination of characters persuade Heemstra and Smith to erect a new suborder (*Hexatrygonoidei*) within the order *Myliobatiformes* to contain this fish. The order contains the other stingrays, eagle rays and mantas, most of which, like *Hexatrygon*, have one or more serrated dagger-like spines on the back of the tail.

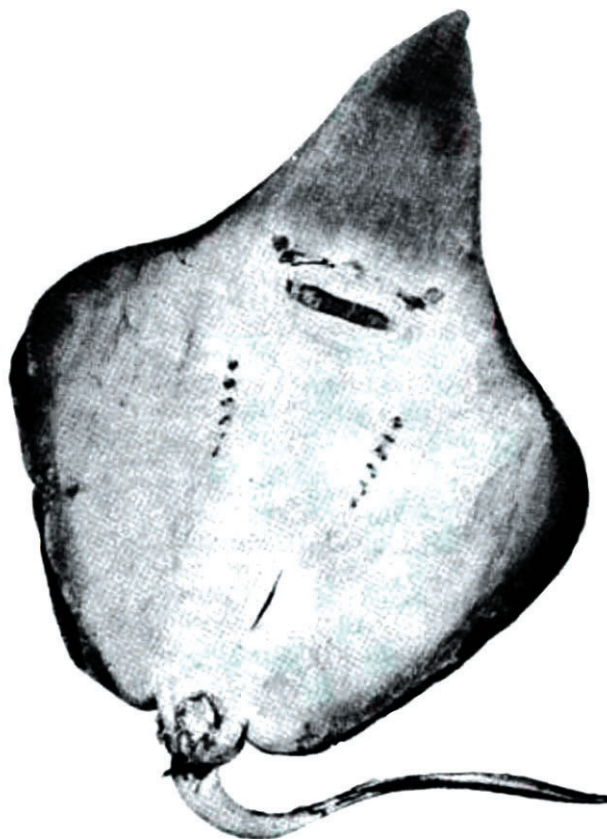
The interest of this fish is not just that it represents a novel group within a well known order, but that all the other myliobatiformes are shallow-water bottom-living species or surface-living fish. *Hexatrygon* seems to be the first deep-water stingray known. Its elongate, jelly-filled snout richly supplied with electroreceptors is similar to the deep-water

chimaeroids *Rhinochimaera* and *Harriota*, and there can be little doubt that it is an adaptation for finding food in the soft-bottom ooze of deep water.

One final point that Heemstra and Smith note is the remarkably small brain of *Hexatrygon* (it is about 3% of the cranial volume); in shallow-water stingrays it is about 80%. As they point out the coelacanth, *Latimeria chalumnae*, has a similarly small brain (as does the basking shark, *Cetorhinus maximus*, according to Scott *J. Fish Biol.* 16, 665; 1980). This may also be an adaptation to deep-water life.

If nothing else the discovery of *Hexatrygon* off South Africa, as with the first coelacanth, reminds one of the tag — *ex Africa semper aliquid novi*.

Alwyne Wheeler is in the Department of Zoology, British Museum (Natural History), London.



Ventral view of *Hexatrygon bickelli*

sediment (apparent age ~8,000 yr)¹⁵. For the advective contribution to vent nutrition, however, the appropriate comparison would be with the ¹⁴C content of suspended particulate organic matter ordinarily present in near-bottom waters, which ought to be somewhere between these extremes, but again, appropriate measurements are not yet available.

In addition to the uncertainties associated with the two initial hypotheses for vent nutrition considered here, a third hypothesis has now entered the scene. Recent evidence (G.N. Somero and H. Felbeck, personal communication) indicates that significant bacterial chemosynthesis may be taking place *outside* the warm-water vents, by symbiotic

microorganisms within the tissues of some of the macro-fauna. Hence, the basic problem of how the hydrothermal-vent fauna gets enough to eat remains an open and challenging question.

We are most grateful to the many colleagues who have shared their ideas with us, as well as those who have given us access to their unpublished data. □

Untangling ataxia-telangiectasia

from Bryn A. Bridges and David G. Harnden

THE rare autosomal recessive disorder, ataxia-telangiectasia (AT), has attracted attention because it is associated with an unusual sensitivity to ionizing radiations and an increased susceptibility to cancer, particularly of the lymphoid tissues. Patients also have a variety of other problems, including a progressive neurological disorder, a substantial immunological deficiency, spontaneous chromosome instability, a defect of DNA repair, premature ageing, raised serum alpha fetoprotein (AFP) levels and other less regular features. How are all these diverse manifestations of the genetic defect interrelated? In an attempt to answer this question and to resolve inconsistencies in reported results, workers from a variety of different fields came together at a recent conference* at the University of Sussex.

Many laboratories have now confirmed the cellular radiosensitivity of AT cells to ionizing radiation and there was agreement that AT cells are also unusually sensitive to the chromosomally radiomimetic anti-tumour agent, bleomycin. Not all agreed that AT cells are sensitive to a range of other chemical mutagens, suggesting that increased sensitivity to any of these agents cannot be very marked although some of the disagreements may be due to differences between AT lines. Three different groups (Paterson, Chalk River; Inoue, Mishima; Jaspers, Rotterdam) all reported complementation between different AT lines for some radiosensitive function and agreed that AT3BI could be distinguished from AT4BI and AT5BI. Attempts to elucidate the molecular basis of the radiosensitivity failed to detect differences between AT cells and normals in the repair of single-strand or double-strand breaks in the DNA using presently available techniques. Two different types of abnormal molecular response were described, a failure to inhibit DNA replication after γ - or X-irradiation, and a defect in one or more repair pathways. A possibly analogous situation exists in *Escherichia coli* where mutations at the *rec A* gene block both inhibition of DNA replication after UV exposure and several pathways of DNA repair. The existence of a repair defect was demonstrated in different ways by several different groups. Cox (Harwell) showed a failure to repair potentially lethal damage in AT cells following X-irradiation. Indirect evidence of a repair defect comes from cytogenetic studies following G_0 irradiation of AT lymphocytes which reveal a large increase in fragments and chromatid aberrations as

compared with normal cells (Taylor, Birmingham). This, together with the observation that caffeine can potentiate DNA damage to visible chromosome damage at least 24 hours after G_0 irradiation of AT cells (Natarajan, Leiden), suggests that there are long-lived unrepaired lesions. The exact nature of the DNA repair defect is still not clear and it is possible that more than one pathway may be affected. Paterson detected a reduction, in some AT cell lines, of γ ray-induced repair replication, and also a deficiency in their capacity to excise and repair specific γ ray-induced base damage, although there has been difficulty in reproducing some of these results elsewhere. Inoue reported a deficiency in cell-free extracts of all AT cell lines studied of an activity which modifies γ ray-damaged 3' sites to permit the action of DNA polymerase. Some, but not all, laboratories could confirm these results.

The other abnormal radiation response which was agreed by several groups is that AT cells show a much less marked decrease in DNA synthesis than do normal cells following γ -irradiation (Edwards, Birmingham; Lehmann, Sussex; Jaspers). Similarly, Scott (Manchester) finds that AT cells do not show the normal post-irradiation mitotic delay. It is not clear how these defects relate to the repair defect.

Unlike the other cancer-prone DNA repair deficiency syndrome, xeroderma pigmentosum, where there is a hypersensitivity of the cultured cells for mutagenesis by UV light, there is no evidence for any spontaneous or radiation-induced hypersensitivity for the induction of gene mutations in AT (Cox; Simons, Leiden). Furthermore, Arlett (Sussex) was unable to detect any radiation-induced gene mutation in AT cells. One of the most interesting features of AT patients is the high level of spontaneous chromosome aberrations in circulating lymphocytes and particularly the tendency of cytogenetically abnormal clones to proliferate in the absence of any haematological abnormality. The formation of these clones following breakage at specific points on chromosome 14 was emphasized and it was suggested (Hecht and McCaw, Tempe, Arizona) that aberrations involving the breakpoint at or near 14q32 may have particular neoplastic potential.

There is definite evidence for a profound immunological defect in AT patients. All have a defect of cell-mediated immunity and some have a deficiency of IgA and IgE. Waldmann (Bethesda) showed that the IgA deficiency is due to lack of synthesis rather than abnormal metabolic rates. There is diminished helper T-cell function, but even stimuli which do not require a helper T function fail to provoke an IgA response, suggesting a defect of B-cell maturation.

Waldmann also presented evidence to suggest that the DNA sequences necessary for IgA production are present in AT patients and there was some speculation about the possibility that the cutting and splicing of DNA required to produce the specific immunoglobulin genes may be impaired. The *rec A* gene of *E. coli* also controls the cutting and splicing involved in genetic recombination. The relationship between the immune defect and the occurrence of lymphoid cancers was considered. Unlike the other immunodeficiency syndromes AT patients show a range of epithelial cell tumours in addition to the preponderance of lymphoid cancers (Spector, Minneapolis). The pattern is also different from that seen in irradiated populations, where there is a preponderance of leukaemias rather than lymphomas. No conclusion was reached, but it seems likely that the cancers in AT are not related in a simple way to either the radiosensitivity or the disordered immune system, but rather to some combination of the two.

The neurological defects in AT patients, particularly progressive cerebellar ataxia, could not at present be readily linked with the biochemical or immune defects. There was, however, evidence that differentiation may be imperfect in many tissues and organs especially gonads, liver, the immune system and the central nervous system and this may in some way be related to the repair defect. The uniformly raised AFP levels in these patients may represent some immaturity of the liver. The deficient thymus appears embryonic rather than atrophic. Kidson (Brisbane) suggested that failure of the nervous system to differentiate properly might in time lead to progressive loss of neurones.

There was a great deal of interest in the possibility of detecting individuals heterozygous for the AT gene. This stems at least in part from the observation that heterozygotes had an increased risk for largely the same spectrum of tumours as the homozygotes (Swift, North Carolina). If current estimates of the AT gene frequency are correct, heterozygotes would comprise one per cent or so of the general population and this would have profound implications. It could be, for example, that AT gene carriers may account for a substantial proportion of early cancers of the lymphoid system. Suggested methods of detecting heterozygotes were low-dose chromosomal radiosensitivity (Kidson) and cell survival under hypoxic conditions (Paterson). The differences between obligate heterozygotes and normals were small, but may be significant in some cases. However, overlap between these two groups means that those tests would not be suitable for the recognition of heterozygotes in the general population.

Bryn Bridges is Director of the MRC Cell Mutation Unit and a Professor at the University of Sussex. David Harnden is Professor of Cancer Studies at the University of Birmingham.

*The workshop was held from November 6th to 7th, 1980, and was organized jointly by the MRC Cell Mutation Unit and the Department of Cancer Studies, University of Birmingham. Sponsorship was provided by the MRC, the Cancer Research Campaign and the International Cancer Research Workshops programme (ICREW) of the UICC.

The emphasis of recent research on AT has been on the sensitivity of these cells to various agents and their effect on DNA synthesis and repair and progress in these areas has been substantial. While these are undoubtedly important, more effort could

in future be directed towards relating these areas to the defect of the immune system and the neurological disorder. Such a relationship, though surprising, must exist since the evidence that AT is due to a single autosomal recessive gene is good. □

previously it had been excluded by competitive pressures. The combination of these pieces of circumstantial evidence led Markgraf to reject the earlier theories of a climatic change which provided the right conditions for *Picea* invasion into Switzerland and to propose that the tree followed in the wake of Neolithic agricultural progress.

One still needs to explain the delay in expansion between Austria and Switzerland. Here Markgraf suggested that the early invasion of *Abies*, *Larix* and *Pinus cembra* (the only central European member of the *Haploxylon* subgenus of *Pinus* and therefore distinguishable on the basis of its pollen) may have formed a dense canopy forest into which *Picea* could not immigrate. All three of these species were present in Austria, but either arrived later or were in lower frequency. It is claimed that such a forest may have a certain degree of inertia which renders it resistant to invasion by new species and also resilient in the face of climatic fluctuations (Smith *Proc. R. Soc. B* **161**, 331; 1965).

Spruce has also moved westwards in Fennoscandia in the latter half of the post-glacial, but here alternative explanations have been proposed. Moe (*Bot. Notiser* **123**, 61; 1970) collected the dates for the rise in *Picea* pollen in this area and showed that its expansion began in eastern Finland by 5,000 BP, reached the Baltic coast of Finland by 3,500 BP and subsequently spread through Sweden and into Norway. It has an unbroken distribution, reaching the west coast of Norway only in the central

The spruce invasion

from Peter D. Moore

ONE of the greatest weaknesses of palaeoecology is its necessary reliance upon circumstantial rather than experimental evidence. Yet it is this weakness which also opens the way to interminable speculation on the basis of the temporal correlation of events in the past. A very good example is the debate concerning the expansion of spruce (*Picea abies*) in Europe since the last glaciation.

Pollen analysis shows that, following the final glacial retreat, *Picea* immigrated into the eastern Alps very rapidly, accompanied by deciduous tree genera such as alder (*Alnus*), hazel (*Corylus*), oak (*Quercus*) and elm (*Ulmus*). It was preceded by birch (*Betula*), pine (*Pinus*) and larch (*Larix*). In Austria, for example, this deciduous forest type expanded into the south-east around 10,200 BP (Fritz *Carinthia* **11** **83**, 277; 1973) and by 9,500 BP it had expanded westwards in the Tyrol (Bortenschlager *Ber. naturw.-med. Ver. Innsbruck* **63**, 105; 1976). Although *Picea* was a member of the early post-glacial forests of Austria, even in the upper valley of the Inn, it is interesting

to note that its immigration into Switzerland was considerably later; and it is here that scope for speculation begins.

Markgraf (*Nature* **288**, 249; 1970) collated the Swiss data concerning the movement of *Picea* into Switzerland and she found that its expansion was not very regular. Only in the south-east had it arrived by about 6,000 BP, but between that date and about 4,500 BP *Picea* pollen increased very markedly in a series of sites scattered all over the country, extending right to the western borders, north of Zurich. There remained some sites, however, even in the south of the country, in which the expansion was delayed until about 3,000 BP or even later. The expansion of spruce was often accompanied by a decrease in other tree species, including fir (*Abies*), pine and the deciduous forest genera. Also there was a simultaneous increase in herbaceous pollen, largely grasses, which suggested that the forests had been disturbed by human activity and that this provided spruce with an opportunity to establish itself where

New vistas in soil research

from Hamish Anderson

FRANCIS BACON'S assertion, that "there is nothing makes a man suspect much, more than to know little", gives an adequate summary of the present state of soil organic chemistry. Soils produce a vast array of organic and inorganic entities — many formed only in the soil environment — which resist synthetic mimicry, long accepted as the ultimate basis of structural proof. There remain few separation techniques or analytical methods which have not been used in humic substance research, and the structural chemistry of soil extractives has uncertainly advanced, mainly by degradative techniques (see Schnitzer and Khan *Soil Organic Matter* Elsevier, 1978).

Proton magnetic resonance spectroscopy is an obvious tool but initial results were beset with line-broadening problems arising from chemical shift anisotropy, even in the rare cases where samples were sufficiently soluble for examination. The advance in NMR capability has now led to the threshold of the soil organic chemist's dream, the possibility of examining humic substances *in situ*

(Barron *et al.* this issue of *Nature*, page 275). The Antipodean researchers have shown that the use of cross-polarization ^{13}C NMR techniques has allowed the generation of useful spectra from whole soil samples. Conventional ^{13}C spectra of amorphous solids usually give no useful structural clues, mainly due to large dipolar interactions. The ^{13}C cross-polarization technique leads to increased sensitivity by the elimination of heteronuclear dipolar broadening, this being achieved by transferring polarization from ^1H to ^{13}C (Mehring *High Resolution NMR Spectroscopy in Solids* Springer-Verlag, 1976). The transfer from the more abundant and more polarized ^1H spins leads to a possible quadrupling of the ^{13}C spins and, more importantly, the ^{13}C spin-lattice relaxation rate now occurs at that of the proton population. Since this is usually an order of magnitude faster than the conventional ^{13}C rate, the data accumulation frequency can be greatly increased. Even allowing for this 'speeding-up' process, it is sobering to realize that the number of free induction

decays collected by Barron *et al.* during whole soil spectrometry ranged from 2×10^4 to 2×10^5 . One major defect of the present system is that the chemical shift anisotropies of aromatic and carboxylic carbon lead to signal broadening and loss of resolution. However Barron *et al.* are confident that the combination of the present high-field cross-polarization technique with 'magic-angle' sample spinning will resolve this problem, as has been demonstrated with isolated humic substances (Hatcher *et al. Org. Geochem.* **2**, 87; 1980).

Humic substances research has long been dogged by the uncertainty of structural alterations occurring during extraction, and the proposed techniques offer answers to this problem. Indeed, the fact that modern NMR spectroscopy offers an ever-widening scope for examining a variety of nuclei instils hope that whole soils and their physical separates can be studied, resolving the interactions between humic substances and silicates, along with the involvement of nutrients such as nitrogen, sulphur and phosphorus.

Hamish Anderson is in the Department of Soil Organic Chemistry, Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen.

region. There are some similarities to the Swiss data in that the expansion is non-synchronous, but in this case there is a more distinct pattern of progressive westward movement.

Tallantire (*Norw. J. Bot.* **19**, 1; 1972) assessed the possible courses of the late post-glacial movements of spruce in Fennoscandia and he regarded the expansion as a step-wise development with phases of expansion, and plots them in this form on his maps. He quotes in full the dates upon which he bases this idea but, if the dates are considered in conjunction with their errors, it cannot be fully supported; it is possible to interpret the spread of spruce as a gradual process. Tallantire considers the expansion a consequence of climatic changes and bases this partly upon his assumption of phased movements, but also upon the inconclusive nature of the palynological evidence for coincident human activity. Further analysis of the climatic requirements of spruce (*J. Biogeogr.* **4**, 219; 1977) led Tallantire to regard the critical parameters as ample early-summer water supply and stable, low winter temperatures. In the absence of either of these factors, the young spruce (2–3 years) is selected against. He concludes "the conformable arrangement of the dates of spruce spread in southwestern Finland and its relationship to present winter climatic data is surely too marked to be due to a chance distribution of the investigated sites."

Since we are dealing with correlations, however, it is worth pointing out that correlation between pollen evidence for human activity and the spread of the spruce is becoming increasingly convincing as more detailed data accumulate, particularly from Finland. In the Kuusamo district of the north-east, *Picea* expanded at the time when tree pollen was falling and increasing Gramineae, *Calluna*, *Vaccinium* and *Empetrum* indicate ground flora changes (Hicks *Trans. Inst. Br. Geogr.* NS1, 362; 1976 and *Commentat. Biol.* **80**, 1; 1975). Although Hicks does not place such an interpretation upon the data, it is possible to support the idea of human agency in *Picea* spread.

Similarly in southern Finland, Tolonen (*Ann. Bot. Fenn.* **15**, 4; 1978 and **17**, 15; 1980) produces pollen stratigraphic curves in which the indications of human activity at the times of *Picea* expansion are strong. Many weed species, in addition to cereals, show short-duration peaks at precisely the time of the *Picea* rise at Lake Lammijärvi. At Lake Ahvenainen, *Picea* pollen rises in a series of episodes at each of which there are also cultural indicators. It is also noteworthy that considerable increases of charcoal are found in the latter site following the horizon at which *Picea* begins its spread.

Tallantire has claimed that the ecological

conditions in Fennoscandia were essentially different from those in central and southern Europe in that *Abies*, *Fagus* and *Carpinus* were not found in the pre-*Picea* woodlands, which consisted largely of *Betula*, *Pinus* and *Sorbus*. These forests would therefore have offered little competitive resistance to *Picea*, it may be supposed. The correlation between forest disturbance and spruce invasion certainly exists, however, and it may be that nutrient release from litter caused by disturbance and especially burning was influential in allowing spruce to invade. In the current

management of Fennoscandian spruce forest, for example, Viro (in *Fire and Ecosystems* (eds T.T. Kozłowski & C.E. Ahlgren), Academic Press, New York, p.7; 1974) recommends the use of fire for rejuvenating old stands.

We are thus faced with the task of balancing alternative correlations, bearing in mind that a strong probability exists that both processes were operative. In the absence of any possibility of experimental confirmation of either hypothesis, the ground is well prepared for lengthy debate and speculation. □

Inclusion compounds

from J.E.D. Davies

COMPOUNDS which show unusual properties, such as a preference for methanol rather than for ethanol, for the *para* rather than for the *ortho* or *meta* isomers of $C_6H_4Cl_2$, for the axial rather than for the equatorial conformer of $C_6H_{11}Cl$, for the *trans* rather than for the *gauche* rotamer of 1-chlorooctane, and even a preference of $C_6D_4(CD_3)_2$ rather than for $C_6H_4(CH_3)_2$, were the subject of a recent symposium* in Poland.

These interesting compounds are the inclusion compounds, which are two-component solid-state systems where the 'guest molecule' is accommodated in voids formed in the crystal structure of the 'host lattice'. These host-guest complexes (for which the term 'hogiplexes' was suggested [J.E.D. Davies, Lancaster]) can be classified according to the type of void available for the guest molecule. The guest species is accommodated in interlayer spaces in the intercalates; in the clathrate compounds the guest species is completely enclosed in a cavity; and the term inclusion compound is usually used where the guest species is accommodated in any other type of void, such as long channels (see figure).

After a historical introduction by W. Kemula (Warsaw) and a general survey of the different types of host-guest complexes by J.E.D. Davies (Lancaster), the symposium heard about current work using a variety of host lattices.

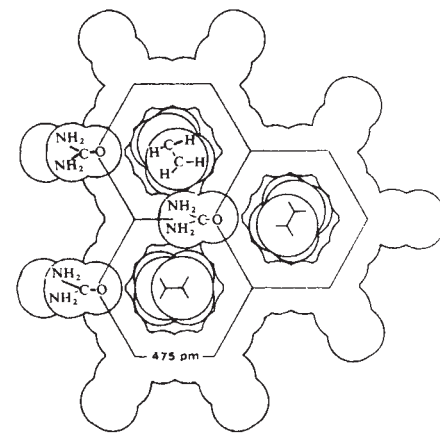
The greatest number of papers was devoted to the Werner-type host lattices, $M(X-py)_4(NCS)_2$, where $M = Mn(II)$, $Co(II)$, $Ni(II)$, $Fe(II)$ and $X = 3-Me$, 4-Me, 4-Et, whose selectivity towards isomeric disubstituted benzenes has long been known (Schaeffer *et al. J. Am. Chem. Soc.* **79**, 5870; 1957) and recent selectivity studies were reported by S. Brzozowski (Warsaw), J.E.D. Davies (Lancaster) and A. Lewartowska (Warsaw). This selectivity can be put to good use, and D. Sybilska (Warsaw)

showed how it is possible to separate isomers chromatographically on columns consisting of the Werner complexes. The chromatographic use of a wider range of inclusion compounds was reviewed by E. Smolkova-Keulemansova (Prague).

Undoubtedly the most unexpected selectivity shown by these host lattices is towards isotopic molecules. N.O. Smith (New York) described his careful work in this area and was able to show that when $Ni(4-Mepy)_4(NCS)_2$ is equilibrated with a mixture of the protonated and deuterated guest, there is a definite selectivity towards the deuterated species with benzene, xylene and naphthalene.

The related compounds, $Ni(NCS)_2(\alpha\text{-arylalkylamine})_4$, also show remarkable selectivities and P. de Radzitzky (Brussels) described the work of the Labofina group on these compounds and illustrated a plant using these compounds to separate the dimethylnaphthalenes on an industrial scale.

Crystallography has been an important technique in the study of inclusion compounds, particularly since it was the pioneering crystallographic studies of H.M. Powell which first elucidated the nature of these compounds, and G.D.



Cross-sectional view of urea-*n*-paraffin complex. (Fig.11 from Smith *Acta crystallogr.* **5**, 224; 1952. Reproduced by permission of *Acta crystallographica*.)

Peter D. Moore is in the Department of Plant Sciences, University of London, King's College.

*First International Symposium on 'Clathrate Compounds and Molecular Inclusion Phenomena' organized by the Institute of Physical Chemistry of the Polish Academy of Sciences and held at Jachranka, Warsaw, 22–26 September 1980. The author acknowledges receipt of a British Council Travel Grant.

The source of meteorites in time and space

from Robert Hutchison

VARIOUS false alarms have occurred in the past over alleged discoveries of fossil meteorites. For example, a squarish piece of iron weighing 785g from Tertiary lignite mined at Wolfsegg, Austria, was originally reported as meteoritic (*C.r. hebdom. Séanc. Acad. Sci., Paris* **103**, 702; 1886; *Nature* **35**, 36; 1887). More recently, in 1930, a fist-sized piece of iron-nickel metal was said to have been recovered from a borehole at a depth of 1,525 feet in Eocene sediments; this 'Zapata County', Texas, iron has since been lost (*Loving Nature* **183**, 1664; 1959). Until the discovery of the remains of a chondrite in Ordovician limestone in Sweden by Thorslund and Wickman (see this issue of *Nature*, p. 285), the only probable fossil meteorite material known to the writer was the occurrence of chondrule-like structures in Mesozoic sediment from the Urals (Yudin *Meteoritics* **6**, 99; 1971). The Ordovician chondrite has been reasonably (but not conclusively) identified as a member of the high-iron or H-group of the ordinary chondrites. Its fall to Earth some 460 Myr ago has considerable significance.

There are compelling arguments that most meteorites come from asteroids. But although it now seems probable that there are sufficient numbers of the Earth-crossing Apollo asteroids and the related

Mars-crossing Amor asteroids to account for the observed flux of meteorites, a steady state can only be maintained if these groups are fed from some source beyond Mars (Wetherill *Geochim. cosmochim. Acta* **40**, 1297; 1976). The small Apollo objects have a mean lifetime of only a few tens of millions of years before they are either captured by a planet or ejected from the Solar System by Jupiter. About 30% of observed meteorite falls are H-group chondrites. Most of these meteorites have cosmic-ray exposure ages close to 4 Myr (see, for example, Zähringer *Geochim. cosmochim. Acta* **32**, 209; 1968), which is taken to mean that they were released as metre-sized objects from some larger body some 4 Myr ago. Release was presumably the result of a collision involving the parent body. No stony meteorite has an exposure age greater than 100 Myr. Thorslund and Wickman's discovery indicates that there was H-group chondrite material among Apollo asteroids some 460 Myr ago, and the possibility then arises that the meteorite flux has not changed significantly in composition with time.

If this is so there appears to be a problem. From spectral reflectance data Gaffey and McCord (*Proc. 8th Lunar Sci. Conf.* **1**, 113; 1977) found that belt asteroids rarely have surfaces with the

mineralogy of ordinary chondrites; such surfaces are common among Apollo and Amor objects. Wetherill concludes that to maintain a steady-state population of these bodies, belt asteroids and, dominantly, comets must have orbits which can evolve into Earth- or Mars-crossing orbits. However, belt asteroids appear to have surfaces which are almost exclusively not ordinary chondritic, and Anders (*Icarus* **24**, 363; 1975) has argued, on the basis of the ancient bombardment undergone by meteorites of different types by particles emitted by solar flares, that most stony meteorites cannot have come from the outer Solar System. The problem of the maintenance of the meteoritic flux was recently discussed by Napier and Clube (*Nature* **282**, 455; 1979), who proposed that a proportion of meteorites come from outside the Solar System, but the meteorites themselves have no major chemical or isotopic anomalies consistent with different regimes of nucleosynthesis; so we are left with a Solar System origin. The question of where in the Solar System that might be has, if anything, been aggravated by the new discovery.

Robert Hutchison is at the British Museum (Natural History), where he is responsible for the curation of the national collection of meteorites.

Andreotti (Parma) dealt with crystallographic studies of a whole range of inclusion compounds. It was therefore surprising to learn of the lack of crystallographic studies on Werner inclusion compounds and J. Lipkowski (Warsaw) reported his recent studies which aimed at trying to understand the factors governing host lattice selectivity. A knowledge of the crystal structure is also necessary to understand the energy-transfer processes between host and guest using UV and γ -ray radiation reported by the group from the Institute of Nuclear Chemistry, Rome. Another new term suggested at the meeting was 'excitrate' (A. Guarino, Rome) to denote a clathrate with either the host or guest, or both, in an excited state.

The Hofmann inclusion compound, $\text{Ni}(\text{NH}_3)_2\text{Ni}(\text{CN})_4 \cdot 2\text{C}_6\text{H}_6$, has been known since 1897, but its properties have only been recently studied extensively. The most prominent worker in this field, T. Iwamoto (Tokyo), described his work on modifying these host lattices by replacing the ammonia by bidentate bridging ligands, and by replacing the square planar $\text{Ni}(\text{CN})_4^{2-}$ group by tetrahedral $\text{M}(\text{CN})_4^{2-}$ groups. These modified host lattices show interesting properties such as the ability of

$\text{CdenHg}(\text{CN})_4$ to stabilize the cyclohexadienyl free radical, produced by the γ -ray irradiation of the benzene guest molecule. This free radical is stabilized up to the thermal decomposition temperature of the host lattice, 423K. Vibrational spectroscopic studies of these systems were reported by J.E.D. Davies (Lancaster) and stability studies were reported by A. Sopková (Košice).

It is true to say that the majority of inclusion compounds known have been discovered by accident, but D.D. MacNicol (Glasgow) gave a fascinating talk outlining his strategy for the synthesis of a variety of compounds which should be expected to act as host lattices. Equally fascinating was the talk by J.L. Atwood (Alabama) describing his work on 'liquid clathrate' systems. These are non-stoichiometric compounds which form in the liquid phase on the interaction of aromatic molecules and $\text{M}[\text{Al}_2\text{R}_6\text{X}]$ moieties. The mole ratio of aromatic to anion can vary enormously, the present record being $[\text{N}(\text{C}_6\text{H}_{13})_4][\text{Al}_2\text{Et}_6\text{I}] \cdot 39.6$ *o*-xylene. A few of these liquid-phase systems have yielded crystalline products, for example $\text{Cs}[\text{Al}_2\text{Me}_6\text{N}_3] \cdot 2p$ -xylene, in which the *p*-xylene molecules sandwich the caesium ion.

A recent interesting application of inclusion compounds is the realization that a chiral host lattice might show enantiomer selectivity, and G. Tsoucaris (Chatenay-Malabry) gave examples of the enantiomeric selectivity of the tri-*o*-thymotide host lattice. Another recent development is the application of solid-state NMR spectroscopy to the study of inclusion compounds. J.A. Ripmeester and D.W. Davidson (Ottawa) illustrated the use of ^{129}Xe NMR in the study of the Structure I xenon clathrate deuterohydrate, where it was possible to observe well separated resonances from the atoms in the two types of cavity.

In conclusion, this was a well timed symposium, as it is apparent that the field of inclusion chemistry has advanced considerably in the 33 years since H.M. Powell first characterized this type of compound, and it is equally apparent that further advances remain to be made. Some of the papers presented at this meeting will be published in a special issue of the *Journal of Molecular Structure*, and it is hoped to hold another symposium in Parma in about two years time. □

J.E.D. Davies is in the Department of Chemistry, University of Lancaster, UK.

Holographic bubble chambers

from David J. Miller

THE new particles have tantalisingly short lifetimes. Even the D^+ , the longest-lived charmed particle, lasts for only about 10^{-12} seconds — time to travel 300 μm at the velocity of light, or a few millimetres with relativistic time dilation. But the D^0 , the Δ_{charm} , the F meson, the τ lepton and the 'beauty' mesons are predicted to travel only some tens of microns before decay. Nuclear emulsions have come back into popularity in the past five years because they gave the only chance of studying such short tracks, and now a new generation of tiny bubble chambers is developing which can resolve bubbles of less than 25 μm in diameter. Each technique has shortcomings — the emulsion requires many scanner-years of work to find the interesting events; a bubble chamber with conventional cameras has such a poor depth of field when the resolution drops below 25 μm that it becomes impossible to focus all of the beam particles into the sensitive volume.

At the Fermilab workshop* Le Coq (CERN) presented impressive pictures and videotapes that had been reconstructed from holograms made with a 6.5 cm diameter by 3.5 cm deep heavy liquid bubble chamber ('BIBC', the Berne Infinitesimal Bubble Chamber). The holographic technique gave clearly resolved images of bubbles only 8 μm in diameter over the whole depth of the chamber. This work has inspired a number of other developments. Pless (MIT) presented scaling rules for the change of resolution when Le Coq's 'straight-through' holographic layout is modified to medium-sized bubble chambers with ~ 1 m diameter. His collaboration is now presenting a proposal to Fermilab for a study of the τ neutrino in such a chamber, using a neutrino beam from a beam dump. A proposal by a European collaboration for a small holographic hydrogen bubble chamber was discussed by Fisher (Rutherford). This would be a continuation of the work being done with conventional optics in proton and pion beams at CERN. He warned that, although the desired resolution can almost certainly be achieved over a useful volume, and although there are techniques available for detecting, measuring and identifying the secondary particles outside the chamber, one serious problem remains for the exploitation of holographic bubble chambers — a very similar problem to that faced by nuclear emulsions. The new particles are produced very rarely — in less

than one event in a thousand. To make an emulsion, or a hologram, really useful, an electronic trigger must be devised which will identify the subsample of events which might contain the short-lived particle. The tracks from these events must then be extrapolated back into the emulsion or the chamber so that the scanner knows where to look. With a bubble chamber it should be possible, in principle, to pulse a laser and make the hologram only when a good event has been detected, but Fisher warned that no technique yet exists which can separate the rare particles cleanly from the background within the few microseconds that it takes for bubbles to grow to the desired size.

Some people think that an alternative approach might bring good results. Instead of looking for a 'needle in a haystack', using small chambers with fine resolution in proton, pion or photon beams, where the backgrounds are enormous, it is perhaps more sensible to work in a neutrino beam where charmed particles occur in 10 per cent of events. To have a reasonable number of neutrino interactions a large bubble chamber must be used, such as BEBC at CERN or the 15 foot chamber at Fermilab. At present these chambers grow their bubbles to around 600 μm in diameter in order to give a clear conventional image from more than 10 m^3 of sensitive volume. A few D^+ tracks have been resolved — the very furthest tail of the length distribution — but many more could be seen if 50 μm resolution were possible. Very little work has been done on the possibilities of using holography in the large bubble chambers but no one has demonstrated that it cannot be done. A number of methods were

suggested: bright field 'Scotchlite' illumination, though work at Imperial College has shown that speckle may be a problem; dark field with Scotchlite, but can sufficiently powerful lasers be found; retrodirecting mirrors, if a suitable reference beam can be set up. Peterson (Hawaii) drew attention to the relevance of these ideas to proposals his collaboration is making for a new chamber at Fermilab. Its primary purpose is to allow secondary particles from neutrino interactions to leave the bubble chamber and be identified, but such a new device could also be designed to incorporate whatever is found to be the most favourable holographic layout.

We were reminded by Lederman, the Fermilab director, that finer resolution means smaller equipment which costs less to build. Fine electronic counters would enable experiments of all kinds to be scaled-down proportionally to the resolution, giving more than proportional reductions in magnet, material and building costs. Such counters were outside the scope of the workshop, but more than one group is convinced that the solution to the 'needle in a haystack' problem will depend on an emulsion or small holographic bubble chamber being followed by electronic counters with a resolution of 20 μm which will both improve the trigger and allow very precise predictions to be passed on to the scanners.

The workshop ended with a theorist's checklist of the effects that could be studied at high resolution. Bjorken (Fermilab) stressed that once the new particles can be collected in reasonable numbers they should show as complex and instructive a phenomenology as did the strange particles. □

D.J. Miller is a lecturer in the Department of Physics and Astronomy at University College London, at present on sabbatical leave in Fermilab.



100 years ago

A "Natural" Experiment in Polarised Light

Break off a plate of ice and hold it between the sky and a pool of water. Its reflected image will show the beautiful colours due to polarised light. The incident rays should come from a part of the sky about 90° from the sun, and reflection should take place at the polarising angle for water, and the plate will probably require adjusting to bring out the maximum effect. Water, vaporous, solid, and liquid, thus furnishes us with polariser, crystal, and analyser. I do not remember to have read any account of this very simple experiment, for which Nature provides all the materials.

Chas. T. Whitmell

9, Beech Grove, Harrogate, January 10

From *Nature* 33, 20 January, 268, 275, 1881.

*A workshop on holographic bubble and streamer chamber techniques was organized by L. Voyvodic at Fermilab, Batavia, Illinois on 11–12 November 1980.

Contaminated cell lines

from David Dickson, Washington

YET another corruption of the scientific literature has been uncovered with the discovery that several cell lines widely quoted in the scientific literature as originating from patients with Hodgkin's disease have now been shown not to be human cells at all, but to come from a strain of owl monkey (see the accompanying article, pages 228–230).

The cells were grown in culture by Dr John C. Long, who resigned a year ago as assistant pathologist at the Massachusetts General Hospital and associate professor of pathology at Harvard Medical School, after admitting to having faked data in a research paper published in the *Journal of the National Cancer Institute* in 1979.

On resigning Dr Long admitted having misled colleagues about carrying out experiments to produce revised data on the molecular weights of immune complexes. The alterations were included in the paper after it had been rejected by the referees of another journal.

Dr Long said on the telephone last week that during his time at the Massachusetts General Hospital, he had no reason to suspect that his cell lines were contaminated and welcomed the work of Harris *et al.* However, when he resigned, he did not reveal that six months earlier he had received data from the National Cancer Institute (NCI) casting doubt on the identity of the cells.

The first successful long-term cultivation of cells from patients with Hodgkin's disease was claimed by Dr Long and colleagues from the hospital and the medical school in *Proceedings of the National Academy of Sciences (PNAS)* in 1973. Other groups have encountered considerable difficulty in establishing permanent lines from diseased spleen cells.

The procedures used to grow the original cells through over 200 passages were described in a 1977 paper in *Journal of Experimental Medicine* as well as *PNAS*. (*Science Citation Index* lists 30 citations of this paper in 1979 and 1980, which its compilers say means the paper is "highly cited".) In total four papers have appeared in *PNAS* — three naming Dr Long as the first author. Experiments using these cells have also been described in other publications (see page 228).

All the papers referring directly to the now-challenged cell cultures will have to be reassessed to see if any data of scientific value can be salvaged, since there is now evidence that the cell lines were contaminated from an early stage.

Dr Long was awarded a three-year grant for more than \$200,000 in 1976 to continue his study of the Hodgkin's disease tissue cultures. This was renewed in 1979 — based on an application said to include the data

which he later admitted forging — and he received \$96,000 in that year, as the first part of a three-year grant of almost \$500,000.

This grant was terminated by the NCI at the request of the hospital, after Dr Long admitted falsifying data in the 1979 paper in *Journal of the National Cancer Institute*. According to the hospital, Dr Long claimed at the time that "competition for federal research grants" was a major factor in causing him to lie about experiments he had not carried out.

However, he did not admit to any other impropriety and the hospital put out a statement saying that the data he had admitted falsifying was "obscure, and of no known significance. . . no known harm has resulted even in the field of science".

The hospital also said that further suspicions about his research were being followed up. There was already evidence of contamination of the cell lines — early in 1979 one of Dr Long's research associates had grown suspicious about the true nature of the cells, and sent samples to Dr Stephen O'Brien at the NCI Laboratory of Viral Carcinogenesis for genotypic evaluation.

The NCI scientists told Dr Long that three of the cell lines carried the enzymatic signature of the same individual. And they also pointed out an unusual characteristic they could not explain — a peculiarly fast mobility in a key enzyme system.

Dr Long did not follow up this comment with the NCI. And he told his research workers that the NCI check had shown that the cells were genuine — indeed that in one case the signature of the cultured cells corresponded directly with that of the red blood cells of the patient from whom the cells were said to have been taken (another claim subsequently shown to be false).

Nothing further happened at the time. But after Dr Long's resignation, Dr Robert T. McCluskey, head of the hospital's pathology department, considered further investigations necessary. Karyotypes and cell samples were sent to the cell culture laboratory at the University of California's School of Public Health, where chromosomal analysis revealed that the cells were not human. Several other laboratories were subsequently sent samples to complete the identification. And the karyotype was recognized as that of the owl monkey by Dr Bharati Hukku of the Child Research Center of Michigan, Detroit.

The cells were then confirmed by the New England Regional Primate Center as coming from a Northern Columbian brown foot owl monkey. Indirect evidence that this was the likely source of contamination was the fact that this cell line had been used for virus research in the early 1970s in the laboratory in which Dr Long was then working.

The fourth suspected cell line, started later than the other three, has turned out to be human. But there is no evidence of a link with Hodgkin's disease tumours.

The full impact of Dr Long's publications remains unclear. His results have been quoted as supporting a particular view of the pathogenesis of Hodgkin's disease which may now be questioned, in particular suggesting a macrophage origin of the peculiarly large Reed-Sternberg cells found in Hodgkin's patients.

Other research workers have been careful not to put too much weight on Dr Long's results, remaining sceptical in the light of their own failure to culture the same cells. And Dr Henry Kaplan of Stanford University feels that the main impact may have been to reduce the incentive for others to tackle the problem of growing the cells *in vitro*, since Dr Long had already claimed success.

Investigators for the Department of Health and Human Services in Boston must now decide whether there is any evidence that should be passed to the Department of Justice to charge Dr Long with fraud against the federal government. Under the US Code, consciously providing false information when applying for federal assistance is a criminal offence.

The journals which published Dr Long's papers now have to decide what action to take. A representative of the National Academy of Sciences has said that the situation was "unique" for *PNAS*, which has never previously had to disown any paper it has published.

Papers submitted to *PNAS* are not formally reviewed if a member of the academy is a co-author. In Dr Long's case, each of the four papers that he published was written with academy member Dr Paul Zamecnik, who recently retired as professor of oncological medicine and director of the J.C. Warren Laboratories at Harvard Medical School, where Dr Long was a research student.

The National Institutes of Health are already discussing how to tighten up on evaluating the scientific content of research funded by federal grants. An audit of Dr Long's grant revealed no fiscal irregularities in the way that the money had been used, but did not go into the legitimacy of the research.

Ironically, the recently created President's Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research is now considering another Boston case, in which a research worker at the Boston University Medical School was shown to have faked data in a cancer survey.

Research administrators admit that cases of fraud — usually relatively minor — are not unusual, although few reach the public eye. "Whatever you see is probably only a small fraction of what actually goes on," according to Dr Donald Kennedy, president of Stanford University.

Contamination of Hodgkin's disease cell cultures

Nancy L. Harris*, David L. Gang*, Steven C. Quay*, Sibrand Poppema*,
Paul C. Zamecnik†, Walter A. Nelson-Rees‡ & Stephen J. O'Brien§

* Department of Pathology, Harvard Medical School, Immunopathology Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

† Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

‡ Cell Culture Laboratory, University of California School of Public Health, Naval Biosciences Laboratory, Oakland, California 94625

§ Laboratory of Viral Carcinogenesis, National Cancer Institute, Bethesda, Maryland 20014

Several laboratories have recently reported the establishment and characterization of long-term cell lines thought to be related to the neoplastic cell of Hodgkin's disease. Here, Harris et al. discuss evidence that some of these lines are, in fact, not related to Hodgkin's disease but are non-human contaminants.

In an effort to determine the origin of the malignant cell of Hodgkin's disease, many investigators have attempted to establish permanent cell lines from tissues involved with the disease. Early attempts to culture tissues involved with Hodgkin's disease yielded either mixed cultures which could not be maintained *in vitro*, or Epstein-Barr virus-infected lymphoblastoid cell lines (see ref. 1 for a review). Recently, several laboratories have reported the establishment and characterization of long-term cell lines thought to be related to the neoplastic cell of Hodgkin's disease²⁻⁷. Our studies indicate that several of these cell lines are not related to Hodgkin's disease, but are non-human contaminants.

Long et al. reported the establishment of four permanent

monolayer cell lines, designated FQ, RB, SpR, and RY, from tumour nodules in spleens of patients with Hodgkin's disease⁷. These four cell lines were considered to be neoplastic by virtue of their capacity for indefinite replication *in vitro*, aneuploidy and the production of invasive tumours on subcutaneous injection into nude mice⁸. Although the cells had no surface markers demonstrable by standard immunological techniques, several features, namely the production of lysozyme and esterases, the ability to phagocytose iron particles⁹ and the ultrastructural morphology of the cells in culture¹⁰, were felt to be consistent with a macrophage origin of the cultured cells. The establishment of these permanent cell lines, which seemed to be neoplastic and had features of macrophages, supported the



Fig. 1 Three marker chromosomes, conventional staining (insert). Trypsin-Giemsa banded metaphase chromosomes of an FQ (top four rows) and an OMK-210 cell (bottom four). The FQ culture exhibited polysomy for many chromosomes including, in some instances, duplicate marker chromosomes. OMK-210 cells (kindly provided by Dr M. Daniel) were near-diploid. Identical marker chromosomes in both cells are indicated by arrows. Most noticeable are isochromosomes for 13q. A small, altered chromosome which resembles the Y chromosome was noted among the markers in the FQ cells [designated Y(?)]. As the karyotype of OMK-210 is female, and the presence of multiple identical markers in the two cell lines indicates a common origin, it is unlikely that the small FQ chromosome is a true Y chromosome. The FQ karyotype was kindly constructed by Dr N. S. F. Ma; its pattern was followed for the OMK-210 cell.

concept that the malignant-appearing cells observed by pathologists in Hodgkin's disease tumours are neoplastic macrophages^{1,9,10}.

Further studies of these cell lines appeared to offer a new insight into the pathogenesis of Hodgkin's disease. It was reported that two of these cell lines (FQ and RB) specifically bound immune complexes from the sera of patients with Hodgkin's disease^{11,12}. As the cells lacked receptors for immunoglobulins and complement and did not bind immune complexes from sera of patients with other disorders, it was concluded that the antibody portion of the immune complex was reacting with an antigen on the cultured cells. This observation not only provided the first direct evidence that the cells cultured *in vitro* were related to the human disorder, but also suggested that some patients with Hodgkin's disease were reacting immunologically to a tumour-associated antigen.

A recurring problem associated with the use of long-term, established cell lines in the study of human tumours is the risk of contamination and overgrowth of cultures by unrelated cell lines¹³. Previous publications have shown that the techniques of trypsin-Giemsa-banded karyotyping¹⁴ and isoenzyme (allozyme) analysis^{15,16} can detect both intra- and interspecies contamination of cell lines. In addition to these methods of genetic characterization of cell lines, the technique of subcutaneous transplantation into nude (athymic) mice can also be used to investigate the origin of a cell line. When noncultured tumour cells or established cell lines are injected into nude mice, the tumours which form may be more differentiated than either the original tumour or the *in vitro* cultured cells, thus permitting determination of the histogenesis of an apparently undifferentiated cell¹⁷.

Using these three techniques, we have found that three of the four cell lines (FQ, RB and SpR) reported by Long *et al.*⁷ are identical to each other and are of owl monkey rather than human origin. Furthermore, they have morphological features of epithelial cells rather than macrophages. The fourth cell line (RY) is of human origin, but its relationship to Hodgkin's disease has not been established.

Cultures of FQ, RB, SpR and RY cells maintained by Dr J. Long, (Immunopathology Laboratory, Massachusetts General Hospital) were studied, as were the earliest available cultures from the laboratory of Dr P. Zamecnik (spanning passages 14-500 of FQ, 300-380 of RB, 21-220 of SPR, and 5-130 of RY). In FQ, RB and SpR cells the chromosome numbers ranged from 75 to 100, with a modal number between 83 and 100 chromosomes. Conventional chromosome preparations of FQ, RB and SpR metaphases revealed three to four copies of a medium-size submetacentric chromosome with a pronounced near-terminal secondary constriction; a chromosome peculiar to normal owl monkey cells (Fig. 1, insert). Trypsin-Giemsa-banded karyotypes of FQ, RB, and SpR exhibited non-human chromosomes, singly or in multiples, and were recognized as polyploid derivatives of cells of the owl monkey, *Aotus tri-virgatus* ($2n = 54$)^{18,19}. Cells at both early and late passages in culture contained many normal chromosomes of the owl monkey karyotype, as well as highly altered, marker chromosomes. Most notable among the latter were one or two isochromosomes involving the long arm of chromosome number 13 (Fig. 1). A search for an owl monkey cell line that was well adapted to *in vitro* culture led to the study of OMK-210 cells, an established cell line derived from owl monkey kidney²⁰. Comparison of FQ, RB and SpR with OMK-210 cells showed that identical marker chromosomes, as well as normal chromosomes, existed in all lines.

Despite the presence of identical marker chromosomes, further chromosomal alterations had clearly taken place following the separation of FQ, RB and SpR from OMK-210 cells, leading to differences in their individual karyotypes. For example, although OMK-210 contained near-diploid numbers of chromosomes, the FQ, RB and SpR cells were all near-tetraploid. Such chromosomal alterations are common occurrences in cultured cell lines²¹.

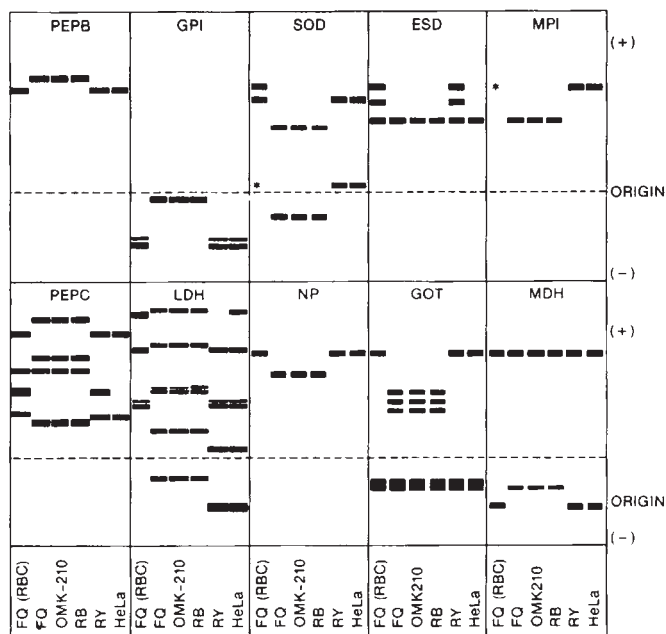


Fig. 2 Composite electropherograms of 10 gene-enzyme systems diagnostic for interspecies cell identification (refs 15, 16). The columns represent: 1, FQ patient's red blood cells; 2, FQ cell line; 3, OMK-210 cells; 4, RB cells; 5, RY cells; 6, HeLa cells. The 10 diagnostic enzymes were: PEPB, peptidase-B (EC 3.4.11.1); GPI, glucose phosphate isomerase (EC 5.3.1.9); SOD, superoxide dismutase (EC 1.15.1.1); ESD, esterase-D (EC 3.1.1.1); MPI, mannose phosphate isomerase (EC 5.3.1.8); PEPC, peptidase-C (EC 3.4.11.1); LDH, lactate dehydrogenase (EC 1.1.1.27); NP, purine nucleoside phosphorylase (EC 2.4.2.1); GOT, glutamate oxaloacetate transaminase (EC 2.6.1.1); and MDH, malate dehydrogenase (EC 1.1.1.37). * Indicates that no activity was observed in that extract for the enzyme. All the systems except ESD vary between man (HeLa) and owl monkey (OMK-210). In man ESD is polymorphic for two alleles *ESD-1* and *ESD-2*. The RY line is heterozygous for *ESD-1-2*. The FQ red cell extract shows that the FQ patient is heterozygous for *ESD-1-2* while the FQ cell has an ESD with a mobility of *ESD-1*, which is identical to that of OMK-210. The OMK-210 isozymes were each identical to those observed in extracts of OMT-1, an owl monkey lymphoid cell line derived by Drs Louis Quattiere and Gary Pearson (data not shown).

The identity of the FQ, RB and SpR cell lines with OMK-210 cells was further established by determining the electrophoretic mobilities of a panel of 10 isoenzymes selected to detect interspecies contamination (Fig. 2). The FQ, RB and SpR cell lines, at the passages examined, were identical to each other and to OMK-210 cells at each locus, and distinct from the human cell line, HeLa, and the red blood cells of the patient from whose spleen the FQ cell line purportedly arose.

Together, the chromosome and isoenzyme analyses indicated that the three supposed Hodgkin's disease cell lines had been cross-contaminated by OMK-210 cells. OMK-210 cells had, in fact, been carried in the laboratory of Drs Long and Zamecnik at the time when the FQ, RB and SpR cell lines were initiated. In the original description of the cell lines, it was reported that the Hodgkin's disease monolayer cultures underwent a morphological alteration *in vitro* after 3-10 months' propagation in culture, with the appearance of polygonal cells that replicate in a mosaic pattern⁸. This appearance of cells with different morphology from that first noted in the primary cultures or early passages is a characteristic occurrence in the course of contamination of cell lines¹⁴.

Subcutaneous tumours formed by the FQ, RB and SpR cells in nude mice contained acinar structures with microvilli and intercellular junctions (Fig. 3a). These features indicated that the cultured cells were epithelial, and not of monocyte-macrophage or lymphoid origin²². The ultrastructural features of the nude mouse tumours contrasted with those of the cells when examined in monolayer culture¹⁰, and indicated that additional differentiation had occurred in the nude mouse. Surface microvilli were present on the monolayer cells, but acinus formation and intercellular junctions were not seen *in vitro*¹⁰. Although a previous report on the transplantation of the FQ, RB and SpR

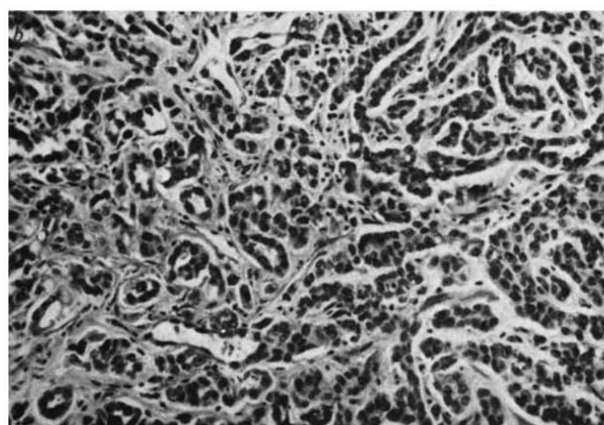
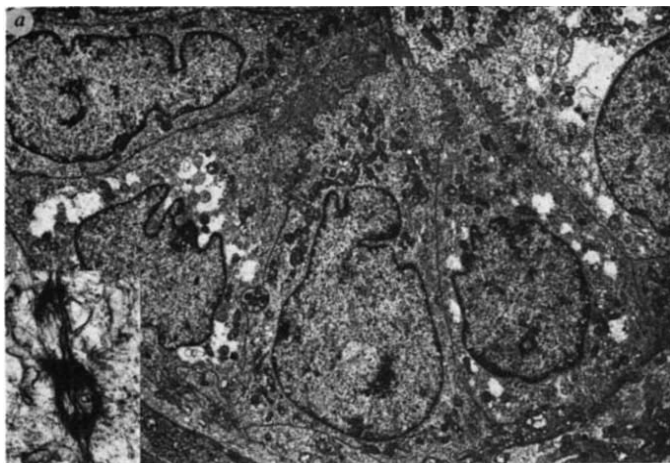


Fig. 3 *a*, Electron micrograph of tumour formed 3 weeks after subcutaneous injection of 5×10^6 FQ cells into a 6-week-old nude mouse (CRL:nu/nu (CD-1) BR, Charles River), fixed in Karnovsky-II and stained by the OCUB method⁹. $\times 27,500$. Insert: intercellular junctions. $\times 27,500$. *b*, Paraffin-embedded section of subcutaneous tumour formed in nude mice by FQ cells (from J. C. Long, as described in ref. 7) showing gland and tubule formation (haematoxylin-eosin, $\times 185$).

cell lines into nude mice stated that the morphology was that of 'reticulum cell sarcoma'⁸, review of the original slides from that study revealed that tubular and acinar structures indicative of epithelial differentiation were present in the tumours formed by these three cell lines (Fig. 3*b*). This observation indicates that contamination of these cell lines had already occurred by then.

The origin of the fourth cell line, RY, could not be established with certainty. The cells had approximately 60 chromosomes. Conventional chromosome preparations did not contain the characteristic owl monkey chromosome with the near-terminal secondary constriction which was seen in the other three cell lines. Trypsin-Giemsa banding revealed an abnormal human karyotype, which was distinct from HeLa or any other established cell line known to us. The isoenzyme genetic signature likewise indicated that the cell line was human (Fig. 2); analysis of a different panel of isoenzymes, selected for their

polymorphism within human populations^{15,16}, confirmed that RY differed from HeLa or any other known cell line (not shown). Tumours formed in the nude mouse by the RY cells, in contrast to the other cell lines, did not have epithelial features; the histogenesis of this cell line could not be determined from the morphology of the nude mouse tumours. As previously described⁷, the RY cell line did not have surface receptors characteristic of macrophages. We were unable to confirm the initial report of lysozyme production by this cell line (S. Quay and S. Poppema, unpublished data). A review of the donor patient's hospital record revealed that the spleen from which the RY cell line originated contained no grossly or microscopically identifiable tumour. As this cell line fails to meet a basic criterion for a tumour cell line—namely, that it be cultured from a specimen known to contain tumour cells²³—its relationship to the malignant cell of Hodgkin's disease remains in doubt.

Although a few other laboratories have reported the establishment of permanent cell lines from patients with Hodgkin's disease, no conclusions regarding the nature of the malignant cell have been drawn from them²⁻⁴. Although there are indications from other sources that the malignant cell of Hodgkin's disease is of macrophage origin, the evidence is not conclusive (for a review, see ref. 24). As the only permanently established Hodgkin's disease cell lines with macrophage characteristics, the cell lines reported by Long *et al.* were thought to provide substantial evidence for the macrophage origin of the Reed-Sternberg cell. Our findings, therefore, remove some of the support for the theory that the malignant cell of Hodgkin's disease is a macrophage. Furthermore, results obtained with these cell lines cast doubt on the specificity of the properties which were considered to indicate that these cultured cells were macrophages. Recently, the RY cell line was found to produce endogenous pyrogen, an observation which it was thought might explain the tendency of some patients with Hodgkin's disease to develop fever²⁵. The significance of this observation must be re-evaluated in the light of our findings.

Our results also indicate that the establishment of permanent cell lines from tissues involved with Hodgkin's disease may be more difficult than had been previously suggested⁶⁻⁹. Several major laboratories have experienced repeated failure in their attempts to establish permanent cell lines from tissues involved with Hodgkin's disease^{24,26,27}. The inability of these other workers to establish permanent lines *in vitro*, despite numerous attempts, may mean that appropriate methods for culture of these cells have not yet been found. On the other hand, it is possible that their very resistance to *in vitro* propagation has some biological significance which has not yet been recognized.

We specially thank Dr Bharati Hukku of the Child Research Center of Michigan, Detroit, who first noted the similarity between certain chromosomes in FQ and *Aotus trivirgatus*. Dr Ward D. Peterson Jr provided additional immunological and serological proof of contamination. Messrs R. R. Flandermeyer and D. W. Daniels and Dr L. Atkins assisted with karyology as did Dr N. S. F. Ma, whose classification of *Aotus* karyotypes was followed. We also thank Drs Robert T. McCluskey, Sigmund A. Weitzman, C. Alan Brown and Clive L. Hall for their support.

Received 10 November; accepted 17 December 1980.

- Kaplan, H. S. *Hodgkin's Disease* (Harvard University Press, Cambridge, 1980).
- Boecker, W. R., Hossfeld, D. K., Gallmeier, W. M. & Schmidt, C. G. *Nature* **258**, 235-236 (1975).
- Roberts, A. N., Smith, K. L., Dowell, B. L. & Hubbard, A. K. *Cancer Res.* **38**, 3033-3043 (1978).
- Schaadt, M., Fonatsch, C., Kirchner, H. & Diehl, V. *Blut* **38**, 185-190 (1979).
- Long, J. C., Aisenberg, A. C., Zamecnik, M. V. & Zamecnik, P. C. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1540-1544 (1973).
- Long, J. C., Aisenberg, A. C. & Zamecnik, P. C. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2285-2289 (1974); **71**, 2605-2609 (1974); *J. natn. Cancer Inst.* **58**, 223-227 (1977).
- Long, J. C., Zamecnik, P. C., Aisenberg, A. C. & Atkins, L. J. *exp. Med.* **145**, 1484-1500 (1977).
- Zamecnik, P. C. & Long, J. C. *Proc. natn. Acad. Sci. U.S.A.* **74**, 754-758 (1977).
- Long, J. C. *Clin. Haemat.* **8**, 531-565 (1979).
- Gang, D. L., Long, J. C., Zamecnik, P. C., Chi, S.-Y. & Dvorak, A. M. *Cancer* **44**, 543-557 (1979).
- Long, J. C. *et al. New Engl. J. Med.* **297**, 295-299 (1977).
- Long, J. C., Dvorak, A. M., Quay, S. C., Stamatos, C. & Chi, S.-Y. *J. natn. Cancer Inst.* **62**, 787-797 (1979).

- Stulberg, C. S. in *Contamination in Tissue Culture* (ed. Fogh, J.) 1-27 (Academic, New York, 1973).
- Nelson-Rees, W. & Flandermeyer, R. P. *Science* **191**, 96-97 (1976); **195**, 1343-1344 (1977).
- O'Brien, S. J., Kleiner, G., Olson, R. & Shannon, J. E. *Science* **195**, 1345-1348 (1977).
- O'Brien, S. J., Shannon, J. E. & Gail, M. H. *In Vitro* **16**, 119-135 (1980).
- Sharkey, F. E. *et al. in The Nude Mouse in Experimental and Clinical Research* (eds Fogh, J. & Giovanella, B. C.) 187-214 (Academic, New York, 1979).
- Miller, C. K. *et al. Cytogenet. Cell Genet.* **19**, 215-226 (1977).
- Ma, N. S. F., Jones, T. C., Miller, A. C., Morgan, L. M. & Adams, E. A. *Lab. Animal Sci.* **26**, 1022-1036 (1976).
- Melendez, L. V. *et al. Lab. Animal Care* **19**, 372-377 (1969).
- Nelson-Rees, W. A. in *Origin and Natural History of Cell Lines* (ed. Barigozzi, C.) 25-79 (Liss, New York, 1978).
- Mackay, B. & Osborne, B. M. in *Pathology Annual* 359-405 (Raven, New York, 1978).
- Fogh, J., Goodenou, M., Loveless, J. & Fogh, H. in *In Vitro Methods in Cell-Mediated and Tumour Immunity* (eds Bloom, B. R. & David, J. R.) 677 (Academic, New York, 1976).
- Kaplan, H. S. *Cancer* **45**, 2439-2474 (1980).
- Bodel, P., Ralph, P., Wenc, K. & Long, J. C. *J. clin. Invest.* **65**, 514-518 (1980).
- Kaplan, H. S. & Gartner, S. *Int. J. Cancer* **19**, 511-525 (1977).
- Sundstrom, C. & Nilsson, K. *Acta path. microbiol. scand.* **86**, 173-184 (1978).

ARTICLES

Multiple asperity model for earthquake prediction

M. Wyss^{*}, A. C. Johnston[†] & F. W. Klein[‡]

^{*} Cooperative Institute for Research in Environmental Sciences, University of Colorado, Boulder, Colorado 80309

[†] Tennessee Earthquake Information Center, Memphis State University, Memphis, Tennessee 38152

[‡] Hawaiian Volcano Observatory, US Geological Survey, Hawaii 96718

Large earthquakes often occur as multiple ruptures reflecting strong variations of stress level along faults. Dense instrument networks with which the volcano Kilauea is monitored provided detailed data on changes of seismic velocity, strain accumulation and earthquake occurrence rate before the 1975 Hawaii 7.2-mag earthquake. During the ~4 yr of preparation time the mainshock source volume had separated into crustal volumes of high stress levels embedded in a larger low-stress volume, showing respectively high- and low-stress precursory anomalies.

OBSERVATIONS of seismic velocity anomalies by Russian seismologists^{1,2} and the development of the dilatancy-diffusion model of earthquake precursors^{3,4}, have encouraged research into earthquake prediction. The simplicity of the microcrack-pore fluid mechanism⁵, its foundation in repeatable observations from laboratory rock mechanics and the absence of contradictory evidence led many seismologists to anticipate in vain that definite prediction of earthquakes would soon be possible. For many medium-size earthquakes ($5 < M < 6$), mostly strike-slip events along the San Andreas fault, the expected precursors could not be detected⁷⁻⁹. These negative findings bring into question the universal applicability of dilatancy to the Earth's crust.

A new model of the preparatory process leading to earthquake ruptures is needed. However, field data for such a model cannot be gathered rapidly and in abundance because we do not know where and when large earthquakes will occur. Without an earthquake prediction capability we may have to wait several decades for large shocks to occur in a location where we can measure in detail the phenomena associated with their preparation process.

The Hawaii earthquake of 1975 occurred within a dense network of geophysical observations. Geodetic and seismological measurements had been carried on systematically for many years to monitor the volcano Kilauea. These data indicate that earthquake precursory changes do occur, but that they show contrasting behaviour in different parts of the earthquake source volume. We interpret this as meaning that the stress level throughout the source volume varies strongly—in small sub-volumes dilatancy-related precursory changes may occur, while strain-softening phenomena (due to stress unloading by creep) occur in other parts of the source volume.

The Hawaii (Kalapana) earthquake

The Hawaii earthquake of 29 November 1975 of 7.2 mag. occurred in the south-east coast of the island of Hawaii on Kilauea's south flank. The rupture occurred along a near-horizontal plane in the Earth's crust at a depth of 8 ± 1 km. Its length was 40–50 km and its width 10–20 km (Fig. 1). Along the south coast subsidence of up to 3.5 m occurred and tsunami heights reached 14.6 m (ref. 6). Damage from the earthquake and related catastrophic events was estimated at \$4.1 million, and two people were killed.

The source of stress in Kilauea's south flank is the intrusion of magma into the volcano and its rifts^{10,11}. The last earthquake of similar size to the 1975 rupture seems to have occurred in 1868. Since then repeated episodic intrusions of magma along the rifts (dotted in Fig. 1) have provided the 'pushing hand' compressing the south flank. The Hawaii earthquake was a rupture of the crust under stress, not a volcanic quake caused directly by an

intrusion. While the stress loading process was different we believe that the rupture process was the same as that in other tectonic earthquakes.

Seismic waves emanating from this rupture were recorded by instruments on Hawaii which are designed to produce on-scale records for the strong ground motions of large, local earthquakes. The seismic energy was radiated in a sequence of high-amplitude pulses separated by comparatively long periods of low-amplitude radiation (Fig. 2). Such records are often observed for large earthquakes and are thought to represent multiple rupture sources¹²⁻¹⁴. As the rupture propagates along a heterogeneous faulting surface, or through a heterogeneous crust, it encounters high- and low-stress volumes. The rupture may speed up and slow down, or stop temporarily, with little radiation emitted from low-stress portions of the fault and large pulses emitted from zones of high stress. The latter may form around asperities on the fault surface, a term which we will use loosely as meaning a strong segment of the fault along which stresses will accumulate.

We conclude that the Hawaii earthquake source volume contained high- and low-stress sub-volumes, based on the strong

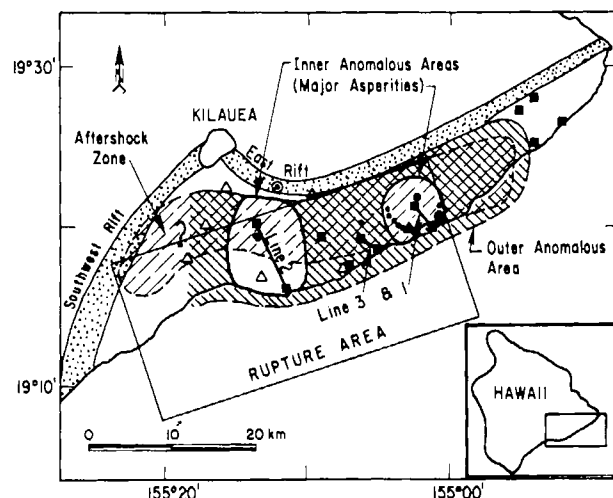


Fig. 1 Map of the south flank of Kilauea volcano showing major geological features, the aftershock zone of the Kalapana earthquake (dashed diagonal hatching), the outer precursor volume (solid hatching), the areas designated as 'major asperities', and the rupture area inferred from surface wave and tsunami data. Δ , Seismograph stations where constant seismic velocities were observed; $\blacktriangle$, the station showing the velocity anomaly; $\blacksquare$, geodetic line benchmarks; $\odot$, the site of the 1972 Mauna Ulu eruption. $\bullet$, Immediate foreshocks; the 5.9-mag foreshock is the larger circle and the 7.2-mag main shock is the largest circle. Some seismic and geodetic stations are omitted for clarity.

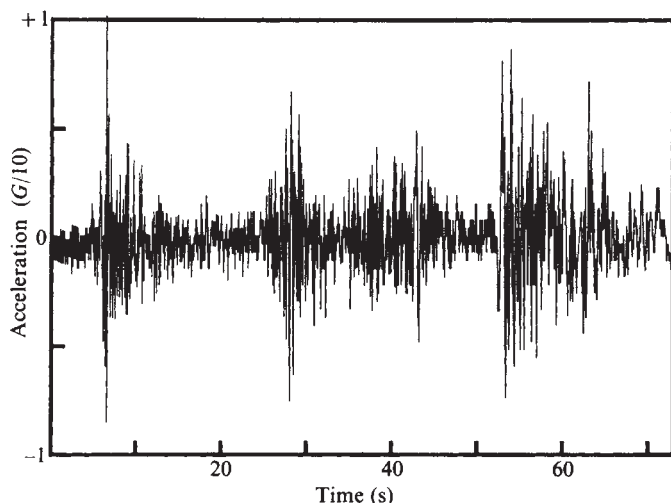


Fig. 2 Strong-motion seismogram of the Hawaii main shock of 29 November 1975, recorded at Hilo. Note that the main rupture seems to be made up of several events emanating energy pulses roughly comparable in amplitude and duration with that of the foreshock²².

motion records (Fig. 2). It is therefore not surprising that in different regions of the source area we found contrasting precursory changes which we interpret as reflecting high- and low-stress regimes respectively.

Precursors to the Hawaii earthquake

The first documented precursor to the Hawaii earthquake was a delay of teleseismic P-wave travel times by 0.2 s lasting from 3.8 yr to several months before the main shock¹⁵ (curve *b* of Fig. 6). According to Student's *t*-test this anomaly is significant at the 99.9% confidence level. Only the station nearest to the epicentre (▲ Fig. 1) exhibited delayed arrivals; six other stations (△, Fig. 1) within 3 km of the rupture area showed no significant change. Nearly horizontal rays from local earthquake sources also showed constant travel times at all stations, indicating that the anomalous volume did not extend to the surface¹⁶.

These observations indicate that the Hawaii earthquake was preceded by a velocity decrease in a volume of ~10-km horizontal extent centred on the hypocentre, while the rest of the source volume remained nondilatant.

The rate of earthquake occurrence may contain information on the state of stress of a crustal volume. If the seismicity rate changes significantly as a function of time, the stress or the strength of the rock in the volume must have changed. Since 1968 all shocks with magnitude 2.0 or more are located routinely. During the 8 yr before the main shock ~1,600 such earthquakes occurred within the main rupture volume. This abundance enabled us to examine separately the rate in sub-volumes of roughly 10-km dimensions. Two types of seismicity behaviour were found. In some volumes the seismicity rate remained approximately constant. An example of this consistency is the seismicity rate of the foreshock volume (eastern major asperity in Fig. 1) as shown in curve 2 in Fig. 3. In other sub-volumes the seismicity rate dropped dramatically around the year 1971 ± 1 (see curve 1 in Fig. 3).

The combined seismicity of the volumes with approximately constant rate (inner anomalous areas of Fig. 1) contrasts sharply with that in the outer anomalous region (Fig. 4) where the rate dropped in early 1972 by ~50% from 120 events yr⁻¹ to 55 events yr⁻¹.

The normal rate in the outer anomalous region is defined by data for the period 1968–71. A longer observation period can be opened if we raise the minimum magnitude in the data set. The seismicity rate in the outer anomalous region for magnitudes 2.4 or more is shown in Fig. 3 (curve 3) for the period 1962–75. Again we see that a highly significant decrease by about 50% occurred. The time of the change in this data set seems to be

late 1970. We conclude that a 50% seismicity rate reduction occurred in the outer anomalous volume in 1971 ± 1 yr and lasted up to the time of the main shock.

Summarizing the geodetic data from 1914 to 1971, Swanson *et al.*¹¹ showed that the south flank was displaced several metres to the south-east with respect to observation points to the north and west of Kilauea's rifts. They also noted that the south flank accumulated compressive strain in the NW-SE direction in steps which coincided with volcanic eruptions and intrusions (Fig. 5). They concluded that shallow (depth < 5 km), forceful magma intrusions under Kilauea and its rifts provided the 'pushing hand' which strained and dislocated the south flank away from Kilauea and the rest of the island. Based on these geodetic data, Swanson *et al.*¹¹ wrote that a large earthquake might be expected on the south flank.

All geodetic observation lines on the south flank showed compression up to 1970. In late 1971 and late 1974 respectively some lines reversed their behaviour, showing extension, that is they indicated strain was released¹⁷. The data for the past eight years are shown in more detail in Fig. 6a. Open circles indicate the average strain registered by lines 1 and 3 (defined in Fig. 1); solid squares show the strain measured on line 2. The strain data interpretation after 1972 is debatable because of the absence of measurements until late 1974. During this period a major phase of the east rift Mauna Ulu eruption commenced (large arrow in Fig. 6; ● in Fig. 1). If the associated intrusive activity of this extrusive event compressed the south flank as observed for previous eruptions¹¹, it could account for the approximate 30-bar increase of line 2 observed in late 1974. Because no data are available for 1972–74, we show in Fig. 6 the uncertainty of the strain (stress) behaviour of this period with both a solid (preferred) and dotted line, depicting two alternative strain histories.

After the late-1974 survey, only line 2 in the western inner region was resurveyed before the 1975 main shock. It revealed a massive, pre-main shock strain relaxation, corresponding to a stress drop of ~30 bar. The earthquakes which occurred during this time could not account for this stress release because they were far too small. We conclude that the strain was released aseptically by some kind of creep process, perhaps along the future fault plane, and at a rate > 10⁻¹² s⁻¹. Significantly, line 2, which accumulated stress up to the end of 1974, was located on a volume of constant seismicity rate, supporting the idea that high seismicity indicates high stress accumulation.

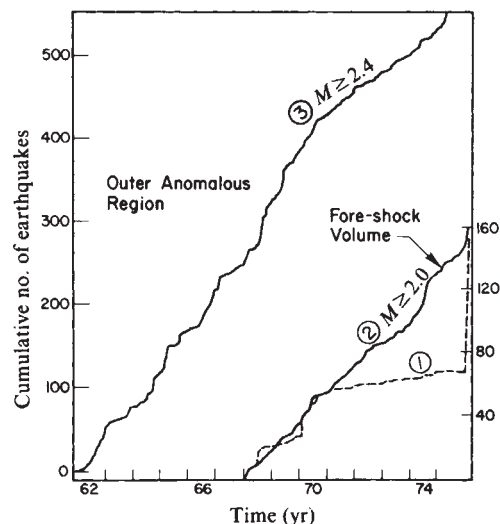


Fig. 3 Cumulative number of earthquakes as a function of time for three subregions of Kilauea's south flank. The slope of the curves equals the seismicity rate. The lower two curves (scale to the right) starting in 1968 are based on earthquakes with magnitudes > 1.9. Curve 1 represents the south flank volume east of longitude 154.9° E, curve 2 the foreshock volume. For a better definition of the normal seismicity rate, the magnitude > 2.3 seismicity from 1962–75 is shown as curve 3 (scale to the left) for the entire, outer anomalous region as defined in Fig. 1.

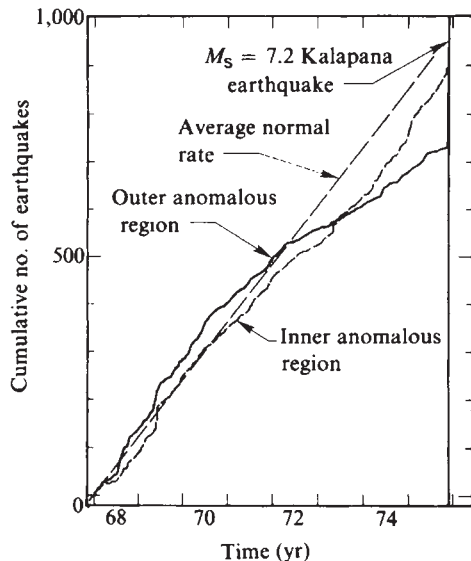


Fig. 4 Cumulative number of earthquakes ($M \geq 2.0$) in the south flank of Kilauea volcano, Hawaii, as a function of time. The solid curve, representing seismicity in the outer precursor volume (see Fig. 1), shows a sharp ($\approx 50\%$) decrease in 1972. The dashed curve indicates roughly constant seismicity rate in the inner precursor volumes.

The strain release along line 1 (1971) and line 2 (1974) is interpreted to indicate that the respective southeastern end points of these lines were then located above the outer anomalous volume. Therefore the outer anomalous volume was apparently expanding at the expense of the inner anomalous volume.

The asperity model

The model for the preparatory process for the Kalapana earthquake must be complex, because different precursory patterns existed in subregions of the future rupture area. The characteristics of the outer anomalous zone are: decreased seismicity rate, strain relaxation and the absence of a travel time anomaly. One or both of the eastern (E) or western (W) inner anomalous zones, exhibited: constant seismicity rate until shortly before the main shock when the rate increased (E and W), a significant P-wave velocity decrease (E), the largest surface displacements during the main shock⁶ (W) and an increase of compressive stress at least up to the fall of 1974 (W).

The decrease of seismicity in the outer volume seems to be directly related to the stress decrease observed there geodetic-

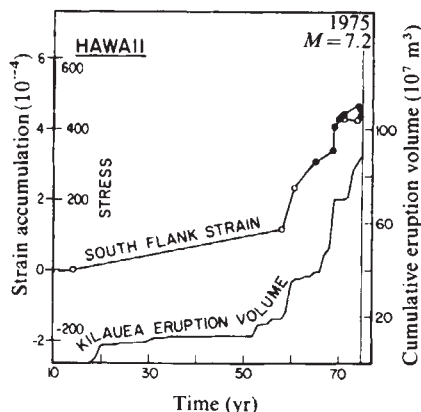


Fig. 5 Strain accumulation on Kilauea's south flank from 1914 to 1975 (upper curve). $\circ$, Averages from the geodetic line 1 and 2 (Fig. 1); $\bullet$, strain along line 2. The cumulative eruption volume is shown for comparison. The strain direction is approximately south-east and the loading cycle is assumed to have started in 1868 after the last large earthquake on the south flank.

ally. Because the microearthquake activity in this region could not have accumulated the 10-bar stress drop throughout the outer anomalous volume, we hypothesize that a seismic fault creep along the nearly horizontal fault plane at 9-km depth caused the strain relaxation and the relative seismic quiescence. The stress decrease also explains the absence of travel time anomalies in the outer volume. The two inner anomalous volumes both have a constant and high seismicity rate. As this contrasts with the observations in the strain-softening outer anomalous volume, we suggest that the high seismicity rate indicates high stress in the inner anomalous zones. This interpretation is supported by different observations in the eastern and western volumes. In the eastern volume, near the point of initial rupture, the travel time anomaly indicates the presence of

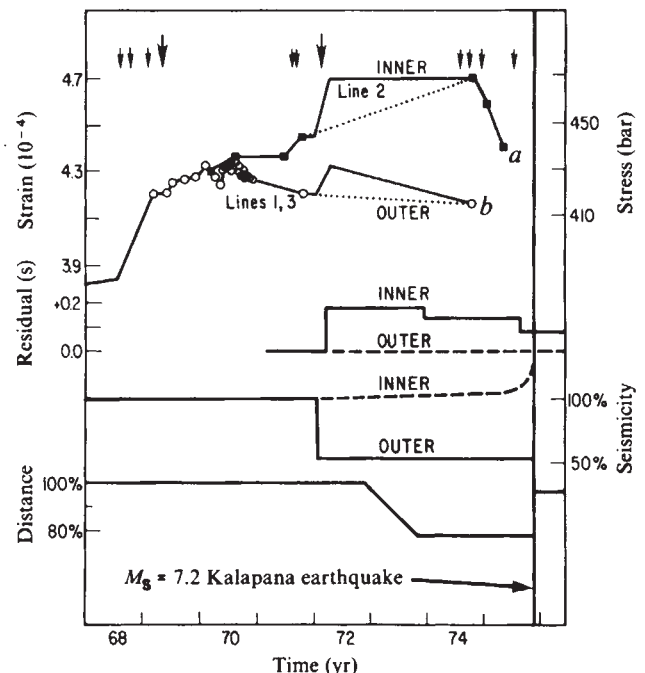


Fig. 6 Schematic summary of crustal parameters during the 8 yr before the Kalapana earthquake. Arrows at the top indicate times and relative size of volcanic intrusive episodes. The top curve shows geodetically measured horizontal strain increase in the western major asperity volume ($\blacksquare$) and strain relaxation in the outer precursor volume ($\circ$). The dotted lines represent a possible alternate strain history between 1972 and 1975 when no measurements were made. The second curve shows the seismic-wave travel-time anomaly observed in the eastern asperity and the absence of such an anomaly in the outer precursor volume. The third curve shows the seismicity rates in the inner and outer precursor volumes (from Fig. 3). The final graph illustrates that the (normalized) average distance of earthquakes from the location of the large foreshock decreased beginning in 1972, which is an alternate way of quantitatively measuring the contrast between seismic activity in the inner and outer anomalous areas. Note that the anomaly onsets of the independent parameters occur at approximately the same time 1971 ± 1 yr. This strongly supports the idea that all of these events are related to the main-shock preparatory process, which may have been initiated by the 1972 large eruption/intrusion event.

dilatancy, which requires high, deviatoric stresses. In the western volume, the stress increase of 30 bar between 1971 and 1974 shows that elevated stresses were present at that time. Thus, our observations indicate that two areas of high stress accumulation exist surrounded by a larger area where aseismic slip is occurring on the future rupture plane. By analogy with rock mechanics studies, we term these small volumes of high-stress concentrations 'major asperities'.

The geodetic line 1 within the eastern asperity showed extension (relaxation) starting in 1970, and the line within the western asperity extended in early 1975 (Figs 5 and 6). This may mean

that these two lines were contained entirely within their asperities until 1970 and 1975 respectively. After these times the creeping portions of the fault plane extended under the respective southern geodetic stations and the associated line began to lengthen. The central and northern parts of the asperities may have been locked until the time of the main shock. Unfortunately there is no geodetic data during the crucial period 1971–74, and the exact time of strain reversal is unknown.

This model of highly stressed asperities embedded in a strain-softening region of lower stress was derived from precursory geophysical data accumulated from the rupture zone of the Kalapana earthquake. It could, however, be considered a corollary of the known multiple-event nature of the main rupture (Fig. 2). Because the main rupture indicates that high-stress volumes were mixed with low-stress ones, the state of stress before the main shock must also have been one of contrasting high- and low-stressed volumes.

Based on laboratory observations, models similar to ours including strain-softening¹⁸ and hard inclusions within strain-softening surroundings^{19,20} have been proposed.

Conclusions

If our model is an accurate representation of the preparatory process to major earthquakes, it may prove difficult to detect high-stress-related earthquake precursors because they may be developed only in small portions of the precursor volume. If our observations can be scaled linearly to smaller and larger earthquakes in other tectonic environments, we expect that for a 5-mag earthquake the major asperities would have radii of ~1.5 km which may explain why no clear velocity anomalies have been detected for such events. For great earthquakes of

several hundred kilometre source length, the asperities might have radii of ~40 km, which means that high-stress precursors might be detected with dense observation networks. The relationships between the number of asperities and earthquake size is not known. Our data do not exclude the possibility that large earthquakes contain numerous asperities with radii smaller than 5 km.

The precursor time for the Kalapana earthquake agrees approximately with the precursor time versus magnitude relations proposed elsewhere^{3,4,21}. We have no evidence for diffusion of pore fluids, so the precursor time will more likely be a function of the ratio of locked-fault area to creeping-fault surface.

We conclude that three independent precursors (Fig. 6) have been observed for the Hawaii earthquake, which constrain the anomaly onset to approximately 1971 ± 1 yr. They are of two types: high-stress precursors (decrease in P-wave velocity and foreshocks) and by contrast low-stress precursors, here observed as a 50% decrease in seismicity and strain relaxation. The high-stress phenomena are restricted to small volumes embedded in a larger volume characterized by lower stresses. The main-shock nucleation point was located in one of the major asperities and the area of largest surface deformations in the other. Our precursor model of hard asperities surrounded by creeping-fault area is analogous to the multiple rupture model for earthquakes. We suggest that future earthquakes may be predicted if patterns similar to those described here are observed.

We thank Robert Koyanagi and the staff of the Hawaiian Volcano Observatory for collecting and organizing data. This work was supported by NSF grant EAR 780-3633.

Received 9 June; accepted 26 September 1980.

1. Savarensky, E. F. *Tectonophysics* **6**, 17–27 (1968).
2. Semenov, A. N. *Bull. Acad. Sci. USSR, Phys. Solid Earth* **3**, 245–248 (1969).
3. Whitcomb, J. H., Garmany, J. D. & Anderson, D. L. *Science* **180**, 632–635 (1973).
4. Scholz, C. H., Sykes, L. R. & Aggarwal, Y. P. *Science* **181**, 803–810 (1973).
5. Nur, A. *Bull. seism. Soc. Am.* **62**, 1217–1222 (1972).
6. Tilling, R. I. *et al.* *U.S. Geol. Surv. Circ. No. 740*, **33**, (1976).
7. Kanamori, H. & Fuis, G. *Bull. seism. Soc. Am.* **66**, 2017–2037 (1976).
8. Bolt, B. A. *Bull. seism. Soc. Am.* **67**, 27–32 (1977).
9. Lindh, A. G., Lockner, D. A. & Lee, W. H. K. *Bull. seism. Soc. Am.* **68**, 721–734 (1978).
10. Ando, M. *J. geophys. Res.* **84**, 7616–7626 (1979).
11. Swanson, D. A., Duffield, W. A. & Fiske, R. S. *U.S. Geol. Surv. Profess. Pap.* 963 (1976).

12. Wyss, M. & Brune, J. N. *Bull. seism. Soc. Am.* **57**, 1017–1023 (1967).
13. Trifunac, M. D. & Brune, J. N. *Bull. seism. Soc. Am.* **60**, 137–160 (1970).
14. Kanamori, H. & Stewart, G. S. *J. geophys. Res.* **83**, 3427–3434 (1978).
15. Johnston, A. C. *Science* **199**, 882–885 (1978).
16. Johnston, A. C. thesis, Univ. Colorado (1979).
17. Lipman, P. W., Lockwood, J. P., Okamura, R. T., Swanson, D. A. & Yamashita, K. M. *U.S. Geol. Surv. Profess. Pap.* (in the press).
18. Mjachkin, V. I., Brace, W. F., Sobolev, G. A. & Dieterich, J. H. *Pure appl. Geophys.* **113**, 169–181 (1975).
19. Brady, B. T. *Pure appl. Geophys.* **112**, 701–725 (1974).
20. Stewart, W. D. *Tectonophysics* **52**, 613–626 (1979).
21. Rikitake, T. *Dev. Solid Earth Geophys.* **9**, 357 (1976).
22. Rojahn, C. & Morill, B. J. *Bull. seism. Soc. Am.* **67**, 493–515 (1977).
23. Wyss, M., Klein, F. W. & Johnston, A. C. *J. geophys. Res.* (in the press).

Upper mantle seismic anisotropy and lithospheric decoupling

J. H. Leven, I. Jackson & A. E. Ringwood

Research School of Earth Sciences, Australian National University, PO Box 4, Canberra 2600, Australia

The lithosphere underlying stable continental shield regions is thought to extend to a depth of ~200 km. Compressional wave velocity models derived from a recent approximately NS refraction profile in northern Australia include a pronounced high-velocity zone at a depth of around 200–250 km. Conventional petrological interpretations in terms of mineralogical or compositional changes cannot readily explain this feature. Calculated velocities for the garnet pyroxene model (using a derived geotherm), fit the velocities above and below this high-velocity zone, but cannot account for the high-velocity (~8.6 km s⁻¹) zone itself. This feature is explained in terms of velocity anisotropy, which might reflect mechanical decoupling of the continental lithosphere from the underlying mantle.

It has been suggested that the lithosphere underlying continental shield regions extends to depths of the order of 200 km, and that the lithosphere (tectosphere)^{1–3} beneath stable continental shields is depleted in low melting point components, is cooler than the normal mantle material at this depth, and hence would translate with the continental plates by virtue of its higher viscosity. Body and surface wave studies of the upper mantle structure beneath shield regions have not indicated any

significant low-velocity zone above a depth of 200 km. A detailed study of the shear wave velocity structure beneath the Australian shield⁴ has not found evidence for any region of low shear wave velocity above a depth of 150 km.

Lehmann^{5,6} first postulated the existence of a seismic discontinuity in the upper mantle at a depth of around 200 km. This feature has subsequently been observed in many upper mantle refraction surveys, commonly at depths shallower than 200 km.

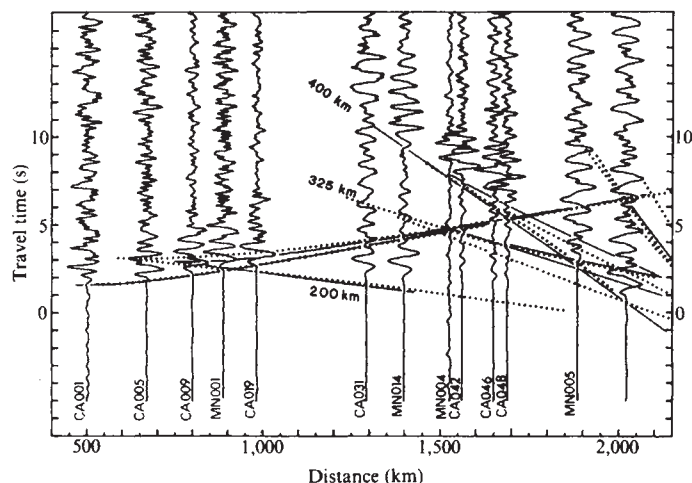


Fig. 1 Vertical component record section of event 256 from the Banda Sea Arc, with the travel time–distance curves of the model 8 (ref. 9) (dotted line) and CAPRI¹¹ (solid line) velocity models superimposed. The branch labelled 200 km corresponds to rays refracted below the 200 km discontinuity. This branch displays a rapid decrease in amplitude beyond an epicentral distance of 1,400 km, corresponding to the onset of a negative velocity gradient beneath the 200 km discontinuity (see Fig. 2).

Anderson⁷ named this upper mantle feature the Lehman discontinuity. Hales⁸ suggested that the relative motion of the Australian continental lithosphere over the underlying mantle material occurs “below a discontinuity in the P-wave velocity at a depth of about 200 km”. Continents can move with comparable velocities to oceanic plates, so where is this zone of relative motion of the continental lithosphere over the underlying mantle and what is the relation, if any, of the ‘200 km’ discontinuity to this zone?

Seismic observations

Hales *et al.*⁹ analysed data from a long range upper mantle refraction profile conducted in Central Australia. Their upper mantle velocity model (model 8) has a velocity discontinuity at a depth of 200 km, with an increase in the compressional-wave velocity of 0.38 km s^{-1} . The depth of this feature has been constrained by the recorded arrivals of an event on the 14 October 1977 (event no. 256). The record (Fig. 1, see also Fig. 14 of ref. 9) of this event, which has been located in the depth range of $176 \pm 5 \text{ km}$ (on the basis of 164 readings), indicates that the 200 km discontinuity is significantly below the 176 km depth. The method of Gutenberg^{9,10} constrains the velocity at the source depth of this event to be $\leq 8.28 \text{ km s}^{-1}$. Beneath the 200 km discontinuity, the velocity is well constrained to be 8.62 km s^{-1} , indicating that the velocity increase across the 200 km discontinuity is $>0.3 \text{ km s}^{-1}$.

The 200 km prograde refraction branch, corresponding to rays which bottom below the 200 km discontinuity, shows an abrupt decrease in amplitude at an epicentral distance of around 1,700 km (depending on the source depth). Figure 12 of ref. 9 shows a fit of the travel time–distance curve derived from their model 5, to the record section for event no. 256. Model 5 did not incorporate any discontinuous increase in velocity around a depth of 200 km, so that this figure indicates the ease with which the 200 km discontinuity can remain undetected, especially when only shallow events are studied. Hales *et al.*⁹ modelled the 200 km structure as a velocity knee with a layer having a negative velocity gradient below. Leven¹¹, using synthetic seismograms to model amplitudes, showed that the amplitude decay of the 200 km prograde branch can be explained by a low velocity zone which starts more sharply, around 30 km beneath the 200 km discontinuity. The exact nature of the velocity profile within the low velocity zone cannot be determined from present refraction data. However, synthetic seismogram modelling indicates that the negative velocity gradient com-

mencing at a depth of 230 km must be sharper than that of model 8 (ref. 9), to explain this rapid decrease in amplitude near 1,700 km distance.

A sharp velocity increase at a depth of around 200 km, followed beneath by a velocity decrease, is a peculiar seismological feature. Similar high-velocity zones in the velocity models of other upper mantle studies can be found—but usually at shallower depths. For example, Lewis and Meyer¹² have a comparable structure at a depth of around 130 km, while in the model of Wiggins and Helmberger¹³, this structure is at a depth of around 150 km. The abrupt decrease in amplitude of the 200 km branch has also been observed in other upper mantle refraction profiles, although the low velocity zone which is implied by this decrease has not been incorporated in the models resulting from these studies. Examples of this abrupt decay in the amplitude of the prograde

200 km branch can be clearly seen in Fig. 8 of ref. 14 and in Figs 11 and 12 of ref. 15. However, no convincing evidence of this type of velocity structure has been observed in the data of any of the Early Rise experiment profiles, which traverse North America with an excellent coverage of azimuth¹⁶.

The existence of a negative velocity gradient at around 200 km depth is supported by the S to P conversion evident in records from teleseismic events. Sacks *et al.*¹⁷ studied these converted phases recorded at the NORSAR array, and have interpreted precursors to the S onset as being S to P conversions from a negative velocity gradient at a depth of $250 \pm 15 \text{ km}$.

Long range refraction surveys for oceanic paths have also indicated a similar velocity structure, but at a considerably shallower depth. Both Asada and Shimamura¹⁸ and Hales *et al.*¹⁹ have an analogous feature in the velocity models derived from oceanic refraction surveys. These velocity models both have a discontinuous increase to a compressional velocity of around 8.6 km s^{-1} , immediately above a low-velocity zone, the depth of this high-velocity zone in both cases being around 75 km. Leeds and others^{20,21} have derived a model for the variation of the lithospheric thickness over the Pacific basin from surface wave studies. Their model indicates a thickening of the oceanic lithosphere from around 76 km in the younger regions, to 104 km in the older regions of the oceanic basin.

Conventional interpretations

Traditionally, seismic discontinuities in the Earth's mantle have been explained in terms of isochemical changes in mineralogy, changes in chemical composition, or a combination of both. The possible relevance of such phenomena to the 200 km discontinuity is now reviewed.

(1) Exsolution of $\text{M}_2^{2+}\text{Al}_2\text{Si}_3\text{O}_{12}$ ($\text{M} = \text{Mg, Fe, Ca}$) garnet from aluminous pyroxenes. The pressure-sensitivity of the Al_2O_3 content of pyroxenes coexisting with garnet provides the basis of pyroxene geobarometry^{22,23}. Along the $1,200^\circ\text{C}$ isotherm, for example, the Al_2O_3 content of orthopyroxene coexisting with garnet in both synthetic and natural systems decreases, rather rapidly from 5–7% near 2.5 GPa to 1.5–2.5% near 4 GPa, and then more gradually to ~0.7% at 7 GPa and 0.4% in Ca-poor clinopyroxene at 14 GPa [ref. 23]. Green and Ringwood²⁴ (see also ref. 25) have shown that the orthopyroxene Al_2O_3 isopleths in the garnet pyroxene stability field are subparallel to the Clark and Ringwood²⁶ Precambrian shield geotherm—suggesting approximate constancy (at the 1.0–1.5% level) of orthopyroxene alumina content over the 2–5 GPa pressure range. The low alumina content of such orthopyroxene and the evidence for its gradual reduction with increasing pressure beyond 5 GPa exclude any possibility that exsolution of pyrope-rich $\text{M}_2^{2+}\text{Al}_2\text{Si}_3\text{O}_{12}$ ($\text{M} = \text{Mg, Fe, Ca}$) garnet from aluminous orthopyroxene is responsible for the discontinuous increase in compressional wave velocity near 200 km depth.

(2) Formation of complex $\text{M}_3^{2+}\text{VI}(\text{Al}_2, \text{M}^{2+}\text{Si})\text{Si}_3\text{O}_{12}$ garnet solid solutions. Ringwood's²⁷ study of the crystallization of a glass of 90% MgSiO_3 –10% Al_2O_3 composition revealed a rapid

increase in both the garnet lattice parameter and the proportion of garnet near 10 GPa. More recently Akaogi and Akimoto²⁸ have confirmed the high pressure solubility of MSiO_3 pyroxene in the garnet structure, but suggest that such solution occurs gradually over the pressure range 4–14 GPa, with a pronounced increase in the proportion of garnet (and hence the density and P-wave velocity) in the 14–19 GPa pressure interval. The fact that the garnet lherzolite nodules of deepest origin (~200 km depth, 7 GPa pressure) brought to the Earth's surface by the Lesotho²³, Kao²² and other kimberlite pipes contain 3.02–3.07 silicon atoms per 12 oxygen atoms is marginal evidence for the initiation of $\text{M}_3^{2+}(\text{Al}_2\text{Si}_3\text{O}_{12} - \text{M}_3^{2+}(\text{M}^{2+}\text{Si})\text{Si}_3\text{O}_{12})$ solid solution near this depth. However, neither laboratory experiments nor the garnet-bearing nodules indicate any abrupt increase in the $\text{M}_3^{2+}(\text{M}^{2+}\text{Si})\text{Si}_3\text{O}_{12}$ content of garnet near 200 km depth in the Earth's mantle. This, coupled with the absence of other mineralogical changes in garnet lherzolite in the relevant pressure range, suggests that phase transformations are not responsible for the rapid increase in P-wave velocity near 200 km depth in the sub-continental mantle.

(3) Compositional change: harzburgite to garnet lherzolite. The long and complex geochemical history of subcontinental uppermost mantle, the geochemistry of ultramafic nodules from kimberlite pipes, and the absence of significant seismic low-velocity zones beneath Precambrian shields suggest that the sub-continental uppermost mantle is likely to be strongly (but variably) stripped of its low melting-point fraction. Under these circumstances, a transition would be expected, perhaps near 200 km depth, from barren harzburgite above, to fertile garnet lherzolite below^{1,25}. The densities and P-wave velocities for these assemblages have been estimated to be 3.31 g cm^{-3} and 8.32 km s^{-1} for harzburgite and 3.38 g cm^{-3} and 8.38 km s^{-1} for pyrolite²⁵. Jordan²⁹ has shown that removal (by 20% partial melting) of the basaltic fraction of pyrolite, decreases the density of the residuum by $\sim 0.05 \text{ g cm}^{-3}$, but leaves the P-wave velocity almost unchanged. The small change in P-wave velocity accompanying such differentiation is due to the near-cancellation between the velocity decrease associated with garnet removal and the velocity increase associated with the more magnesian composition of the residual olivine and orthopyroxene²⁹.

Clearly, although the subcontinental upper mantle must be chemically zoned as a result of differentiation, it is virtually impossible that a P-wave velocity discontinuity of the order of 0.3 km s^{-1} near 200 km depth, can be explained in these terms.

(4) Compositional change: garnet lherzolite (or harzburgite) to eclogite. Anderson^{7,30} has suggested that the Lehmann discontinuity might represent a change in chemical composition from garnet lherzolite (or harzburgite) above the discontinuity to eclogite below. Such eclogite, whether produced by subduction of oceanic basaltic crust (as in Anderson's model), or by the necessarily relatively slow crystallization of basaltic magmas within the mantle, would be expected to possess an average mid-ocean ridge basaltic Mg number ($100 \text{ Mg}/(\text{Mg} + \text{Fe}^{2+})$) of about 60 (see refs 31, 32). In the former case, the basaltic major element chemistry might be perturbed during subduction by partial melting in the quartz eclogite stability field, resulting principally in significantly increased $(\text{MgO} + \text{FeO})/\text{CaO}$ in the residuum (see ref. 33). Typical mantle-derived eclogites are quartz-free, essentially biminerallitic aggregates of pyroxene and garnet (in approximately equal proportions) with Mg numbers around 60.

Mean compressional-wave velocities for such mantle-derived eclogites^{34–36} (samples 11, 14, and 15 of ref. 36 but not 8 which is very magnesian (Mg number 71) and contains 74.0% clinopyroxene, 16.6% garnet, and 7.9% zoisite) span the range $8.27\text{--}8.50 \text{ km s}^{-1}$ at 1.0 GPa with measured velocity anisotropies of 0.8–3.4%. Shear-wave velocities are more variable ($4.49\text{--}4.86 \text{ km s}^{-1}$ at 1.0 GPa) with anisotropies of 1.5–4.9%. The Nové Dvory (Czechoslovakia) eclogite studied by Christensen to 3.0 GPa is representative of the highest P-wave velocity eclogites with $\rho = 3.56 \text{ g cm}^{-3}$, and extrapolated

compressional and shear wave velocities of 8.33 and 4.59 at atmospheric pressure. Our preferred parameters for garnet pyrolite are $\rho = 3.40 \text{ g cm}^{-3}$, $V_p = 8.31 \text{ km s}^{-1}$, and $V_s = 4.82 \text{ km s}^{-1}$ (these differ slightly from those of Jordan²⁹ because of our use of the pyrope elastic moduli measured by Leitner *et al.*³⁷).

Our analysis thus strongly reinforces other observations^{7,25,34} concerning the indistinguishability of garnet lherzolite and eclogite P-wave velocities. We conclude that a change in chemical composition from garnet lherzolite to eclogite might produce a significant decrease in shear wave velocity but that no discernable change in compressional wave velocity would be expected. Under these circumstances, it seems unlikely that the Lehmann discontinuity reflects a chemical change of this type.

(5) Base of pronounced low-velocity zone. Many seismic models for upper mantle velocities beneath tectonically active continental regions (such as the western US) include a substantial and rapid increase in seismic wave velocities in the depth range 150–200 km (refs 38, 39). In most such models, the discontinuity represents the base of a pronounced low-velocity zone, so that a large part of the velocity contrast across the discontinuity is accounted for by a return to 'normal' upper mantle velocities (for example, at substantially subsolidus temperatures) below the discontinuity. The absence of significant low-velocity zones, shallower than 200 km depth, beneath Precambrian shield regions precludes such an interpretation for the Lehmann discontinuity.

Seismic-wave velocities for garnet pyrolite

The above arguments demonstrate the difficulty of accounting for the observed 0.3 km s^{-1} increase in V_p at ~200 km in terms of conventional explanations. To understand the nature of the seismic feature near 200 km depth, we now compare the CAPRI velocity-depth model¹¹ with calculated P-wave velocities for an assumed garnet pyrolite upper mantle.

For this calculation, we assume that the compressional-wave velocity (V_p of $8.31 \pm 0.05 \text{ km s}^{-1}$ at STP) varies linearly with pressure (to ~10 GPa) and temperature (to ~1,400 °C). Because volumetric strains will be no greater than 8% at 10 GPa, finite strain equations of state should not be necessary. The general tendency for both pressure and temperature derivatives of the elastic-wave velocity to decrease with increasing pressure and temperature suggests that linear extrapolation, using derivatives measured near STP, may result in slight overestimation of velocities at the upper end of the extrapolation. Any relaxation of ultrasonically determined shear moduli for upper mantle seismic-wave propagation would similarly result in an overestimation of the seismic velocities.

The pressure and temperature derivatives, used in the calculation:

$$\partial V_p / \partial P = 0.106 \text{ km s}^{-1} \text{ GPa}^{-1}$$

$$\partial V_p / \partial T = -0.55 \times 10^{-3} \text{ km s}^{-1} \text{ K}^{-1}$$

represent a weighted average (57% olivine, 17% orthopyroxene, 12% clinopyroxene, 14% garnet) of the mineral pressure and temperature derivatives. Data for Stillwater bronzoite ($\partial V_p / \partial P = 0.137 \text{ km s}^{-1} \text{ GPa}^{-1}$ (refs 35, 40)), are used in preference to the much higher value $\partial V_p / \partial P = 0.207 \text{ km s}^{-1} \text{ GPa}^{-1}$ derived from a study of single-crystal bronzoite⁴¹. The latter value is grossly inconsistent with the expectation⁴² that $(1/V_p)(\partial V_p / \partial P)$ is approximately equal to

$$\frac{39K + 16\mu}{6K(3K + 4\mu)}$$

This relation yields an expected $\partial V_p / \partial P$ of $0.138 \text{ km s}^{-1} \text{ GPa}^{-1}$, in agreement with the measured value for Stillwater bronzoite. Furthermore, even if the unusually large single-crystal pressure derivatives ($dK/dP = 9.6$) were, in fact, appropriate at STP, it seems improbable that such a high derivative would be maintained over a substantial pressure range. Use of Frisillo and

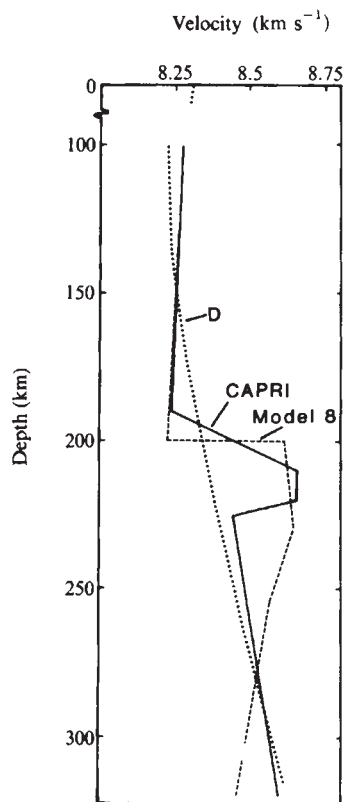


Fig. 2 A comparison of the model 8 and CAPRI compressional-wave velocity–depth models with calculated velocities (*D*) for a garnet pyroxene upper mantle based on the geotherm presented in the text.

Barsch data for orthopyroxene⁹ would increase the garnet pyroxene $\partial V_p/\partial P$ to $0.128 \text{ km s}^{-1} \text{ GPa}^{-1}$ and hence raise the velocity at 200 km depth by $\sim 0.14 \text{ km s}^{-1}$. Further experimental work on single-crystal and polycrystalline orthopyroxene is needed to resolve this uncertainty. Olivine and garnet pressure and temperature derivatives were taken from refs 43–45. A clinopyroxene ($\partial V_p/\partial P$) of $0.119 \text{ km s}^{-1} \text{ GPa}^{-1}$ was obtained by comparing Christensen's³⁵ data for Nové Dvory eclogite with the Bonczar *et al.*⁴⁵ data for garnet. Otherwise, it was assumed that the orthopyroxene derivatives also apply to clinopyroxene. In the initial calculations we used the geotherm of Harte⁴⁶. The resulting compressional-wave velocity profile was broadly consistent with the model 8 of Hales *et al.*⁹ and the CAPRI model in the 75–190 km and 225–320 km depth ranges but failed to explain the high velocities in the 190–225 km interval. Variation of the geotherm within reasonable limits²⁹ allowed a more satisfactory fit (Fig. 2) to the CAPRI velocities in the 75–190 km and 225–320 km depth ranges. The required geotherm

depth (km):	100; 150; 200; 250; 300
temperature (°C):	770; 1,050; 1,180; 1,320; 1,420

is between, and qualitatively similar to, Jordan's²⁹ S1 and S2 conductive geotherms, and would intersect adiabats for the oceanic upper mantle at depths between 300 and 400 km.

The comparison presented in Fig. 2 implies that the garnet pyroxene composition and mineralogy, combined with a reasonable geotherm, explains compressional-wave velocities for all but a narrow interval (190–225 km) of the 75–300 km depth range. Similar difficulties in explaining high velocities just below 200 km are evident in the studies of Graham⁴⁷, and of Ahrens⁴⁸ who noted that none of his calculated models "predict the 8.65 km s^{-1} P-velocity maximum . . . at 200 km depth in profile HWNE" (of Wiggins and Helmberger¹³). There have been similar comments⁴⁹ on the difficulty of explaining compressional-wave velocities $\geq 8.5 \text{ km s}^{-1}$ in some seismic models of the

oceanic lithosphere, in terms of the calculated elastic properties of petrologically appropriate isotropic aggregates.

The match between the calculated velocities and the CAPRI model, other than in the 190–225 km depth range, precludes neither a more barren harzburgitic chemistry above 200 km, nor a gradual increase in the $\text{M}_3^{2+}(\text{M}^{2+}\text{Si})\text{Si}_3\text{O}_{12}$ component of the garnet with increasing depth below 200–250 km.

Seismic velocity anisotropy?

We are clearly concerned, not with a seismic discontinuity in the usual sense, but rather with a zone of the order of 50 km thick, characterized by higher velocities than both overlying and underlying mantle.

We have demonstrated the difficulty involved in explaining the 0.3 km s^{-1} velocity increase near 200 km, in terms of mineralogical or chemical changes. The negative velocity gradient below 230 km is also not readily explained in conventional terms. Leven¹¹ inferred that this velocity gradient must be more negative than $-0.002 \text{ km}^{-1} \text{ s}^{-1} \text{ km}^{-1}$ to produce synthetic seismograms consistent with the observed rapid decrease in first arrival amplitudes near $\Delta = 1,700 \text{ km}$. Using P-wave velocity pressure and temperature derivatives appropriate for garnet pyroxene, we find that an unusually large (but not impossible) temperature gradient, of at least 9°C km^{-1} , would be required if the negative velocity gradient below 230 km depth⁹, were to be explained in terms of the combined effects of pressure and temperature.

On the other hand, this sub-continental 'high-velocity zone' bears a strong qualitative resemblance to shallower high-velocity lids capping low-velocity zones in many velocity models for the sub-oceanic upper mantle. These considerations, and the variability of its observation suggest that this unusual seismic feature might be explained in terms of seismic velocity anisotropy associated with deformation-induced preferred orientation of olivine (and possibly orthopyroxene) crystals in a shear zone, at 200–250 km depth, which is responsible for decoupling of the continental lithosphere from the underlying mantle. This hypothesis is supported by the following observations.

(1) Extensive field and laboratory studies of deformed peridotites from the Lanzo massif in Northern Italy (believed to represent subcontinental mantle), have established a firm relationship between the observed 7% velocity anisotropy, the fabric and the flow coordinates^{50,51}. The velocity anisotropy is associated with preferred orientation of the [100] axes of olivine crystals at a low angle of inclination ($\theta = 10^\circ$, Fig. 3) to the flow direction during high temperature ($\sim 1,200^\circ \text{C}$) deformation at an inferred strain rate of 10^{-14} s^{-1} .

(2) Experiments conducted over a wide range of temperature and strain rate^{51–54}, have demonstrated the development of strong preferred orientation of olivine ($[010]/\sigma_1$ and $[100]/\sigma_3$) as a consequence of laboratory deformation of dunite. At high temperatures and moderate strain rates (moderate flow stress), the preferred orientation seems to be derived primarily from syntectonic recrystallization driven by strain accumulation, due principally to dislocation glide on the system (010)[100]. The coincidence of principal axes of stress and strain in most laboratory deformation experiments^{52,54} results in an ambiguity concerning predicted fabric orientation in natural triaxial deformations which may resolve the apparent conflict between the orientations of the natural and laboratory fabrics mentioned above.

(3) Studies of the azimuthal variation of compressional-wave velocities in the oceanic upper mantle^{55,56} have established the velocity anisotropy of the sub-oceanic mantle. The fast direction in each case is sub-parallel to the spreading direction, that is normal to the mid-ocean ridge. The azimuthal range in P-wave velocities ($\sim 0.3 \text{ km s}^{-1}$) and the association of fast velocities with the direction of plate movement over underlying mantle are both appropriate for explanation of the high P-wave velocities at 200–250 km depth along a NS profile beneath the Australian plate (Fig. 3).

(4) The seismic implications of the development of such an olivine preferred orientation are more fully explored in Fig. 3. For small inclinations of the fast [100] axis to the flow direction (N), propagation directions within 45° of the flow direction display compressional (or quasi-compressional) mode velocities significantly higher than 8.42 km s^{-1} which is the Voigt-Reuss-Hill (VRH) average. Note that the considerable shear wave birefringence—the difference in quasi-shear mode velocities is as much as 0.7 km s^{-1} for some propagation directions. Thorough characterization⁵⁷ of an essentially monomineralic sample of Twin Sisters dunite with a strong preferred orientation revealed a high degree of seismic anisotropy ($7.98 \leq V_p \leq 9.24 \text{ km s}^{-1}$, $4.57 \leq V_s \leq 5.01 \text{ km s}^{-1}$) and shear wave birefringence (maximum $\delta V_s = 0.38 \text{ km s}^{-1}$). In upper mantle garnet peridotite, the magnitude of these effects will be reduced because of the reduced proportion of olivine. Nevertheless, the results of Fig. 3 provide a firm semiquantitative basis for testing the preferred orientation hypothesis.

The model provides a qualitative explanation of the high compressional-wave velocities for approximately NS propagation at depths of 200–250 km in the Australian upper mantle. The results presented in Fig. 3 (see especially $\theta = \phi = 22.5^\circ$)

suggest that significant shear wave birefringence might also be observed (on both horizontal and vertical components) at appropriate azimuths.

(5) Both the chemistries and textures of deep-seated ultramafic modules contained in kimberlite pipes are consistent with the view that decoupling of the continental lithosphere and the underlying mantle is concentrated at depths near 200 km (ref. 58). The observation that ultramafic nodules of deepest origin ($\sim 200 \text{ km}$), brought to the surface by South African kimberlites varying in age by more than 1,000 Myr (refs 22, 59) display more primitive chemistry than their shallower counterparts, suggests that the former are derived from a zone of the mantle which is continuously replenished from a large mantle reservoir. Furthermore, while the highly sheared mosaic textures of the deepest-seated xenoliths are probably not representative of steady-state mantle deformation processes⁶⁰, the contrasting fabrics of these and the relatively undeformed coarse-grained xenoliths⁴⁹ of shallower origin, are suggestive, at least, of very different modes of xenolith incorporation and magma transport.

(6) The combination of more primitive chemistry below 200 km depth and the general characteristics of upper mantle geotherms suggests that the zone between 200 and 300 km depth may be characterized by higher homologous temperatures (T/T_m) for olivine than for the shallower and the deeper mantle. Under these circumstances, this region is a plausible zone for strain concentration, although the relative roles of dislocation creep—syntectonic recrystallization (which may be subject to strain hardening) and grain boundary sliding⁶¹, remain to be determined. Anderson³⁰ similarly concludes that the viscosity of subcontinental mantle should attain a minimum value near 200–250 km depth.

Conclusions

We have demonstrated the difficulty involved in explaining high compressional-wave velocities for approximately NS propagation at 200–250 km depth in the Australian upper mantle in terms of various suggested variations of mantle mineralogy and chemistry. Having rejected conventional interpretations, we suggest that the observed high P-wave velocities may derive from preferred orientation of olivine crystals in a deformation zone responsible for the mechanical decoupling of the lithosphere from the underlying mantle.

Refraction profiles of the Australian upper mantle^{9,62–64}, while in general agreement on the need for a substantial increase in compressional-wave velocity near 200 km depth for profiles within 45° of NS, provide limited information for other azimuths. The only indication of a possible 200 km discontinuity on an approximately EW profile is provided by a single first arrival⁶⁵ at $\Delta \sim 2,100 \text{ km}$ (Meekatharra, Western Australia) from an explosion at Mt Fitton (South Australia). This datum, while possibly compatible with NS profiles, does not provide a strong independent constraint on the velocity below 200 km depth for EW propagation azimuths. Clearly, the anisotropy hypothesis needs further testing. Carefully controlled experiments over a wide range of azimuths (such as those conducted on the suboceanic mantle⁵⁶) will be required to test for anisotropy and possible shear wave birefringence (see ref. 66) of the continental upper mantle.

However, observation of subcontinental upper mantle seismic anisotropy may be subject to considerable variability. Spatial variation in the abundances of key volatile species (such as H_2O), and of mantle geotherms and strain rates may result in variability of the dominant upper mantle deformation mechanism, with some plausible flow mechanisms (such as grain boundary sliding) having little tendency to produce a strong preferred orientation. Even where an appropriate mechanism (such as dislocation creep—syntectonic recrystallization) dominates, the development of an observable seismic anisotropy may depend on a long, consistent and current history of relative motion of the lithospheric plate with respect to underlying mantle.

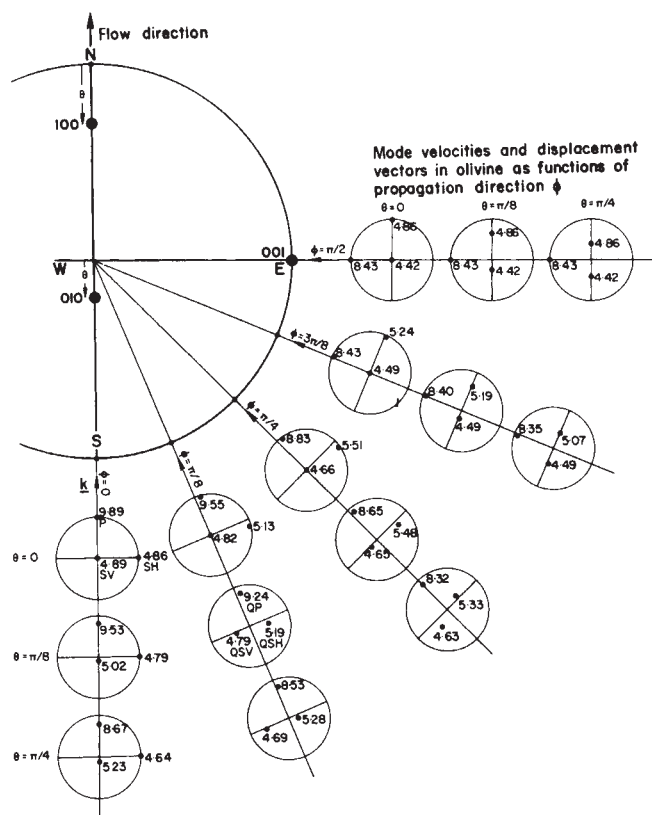


Fig. 3 Seismic anisotropy and shear-wave birefringence of olivine. The crystal orientation is the expected preferred orientation of olivine (see text) in the Australian upper mantle: flow plane horizontal, plate movement over underlying mantle due North. The olivine c -axis (001) lies EW while the inclination θ of the a -axis (001) to the flow direction is varied between 0 and $\pi/4$ (see text). Mode velocities and displacement vectors for horizontal propagation vectors k are calculated as functions of azimuth ϕ and inclination θ from the single crystal moduli⁴³. The particle displacement vectors for the three independent propagation modes for each (ϕ, θ) pair are plotted (●) on a stereographic projection and labelled with the appropriate velocities (in km s^{-1}). Note that off-axis propagation directions involve modes for which particle displacement is neither parallel nor orthogonal to the propagation vector. Such coupled modes are labelled QP, OSH, QSV⁶⁷. The results may be applied in the SW quadrant by virtue of the mirror symmetry of olivine about the plane normal to 001.

Such anisotropy beneath both continental and oceanic lithosphere, would facilitate seismic mapping of the vertical extent of the lithosphere, and provide considerable insight into the rheological behaviour of the upper mantle.

We thank P. N. Chopra, D. A. Gust, A. L. Hales, and K. J. Muirhead for useful discussion.

Received 26 June; accepted 11 November 1980.

1. Ringwood, A. E. *J. geophys. Res.* **67**, 857–867 (1962).
2. Jordan, T. H. *Rev. Geophys. Space Phys.* **13**, 1–12 (1975).
3. Jordan, T. H. *Nature* **274**, 544–548 (1978).
4. Hales, A. L., Muirhead, K. J. & Rynn, J. M. W. *Geophys. J. R. astr. Soc.* (submitted).
5. Lehmann, I. *Ann. Geophys.* **15**, 93–118 (1959).
6. Lehmann, I. *Bull. seism. Soc. Am.* **52**, 519–526 (1962).
7. Anderson, D. L. *J. geophys. Res.* **84**, 7555–7560 (1979).
8. Hales, A. L. *Director's Report, Research School of Earth Sciences Annual Report* (Australian National University, Canberra, 1977).
9. Hales, A. L., Muirhead, K. J. & Rynn, J. M. W. *Tectonophysics* **63**, 309–348 (1980).
10. Gutenberg, B. *Bull. seism. Soc. Am.* **3**, 223–232 (1953).
11. Leven, J. H. thesis, Australian National University (1980).
12. Lewis, B. T. R. & Meyer, R. P. *Bull. seism. Soc. Am.* **58**, 565–596 (1968).
13. Wiggins, R. A. & Helmberger, D. V. *J. geophys. Res.* **78**, 1870–1880 (1973).
14. Masse, R. P., Landisman, M. & Jenkins, J. B. *Geophys. J. R. astr. Soc.* **30**, 19–36 (1972).
15. Johnson, L. R. *J. geophys. Res.* **72**, 6309–6325 (1967).
16. Warren, D. H. *et al. Project Early Rise, Travel Times and Amplitudes* (U.S. Geological Survey Open File Report, Menlo Park, 1968).
17. Sacks, I. S., Snoke, J. A. & Husebye, E. S. *Tectonophysics* **56**, 101–110 (1979).
18. Asada, T. & Shimamura, H. in *The Geophysics of the Pacific Ocean Basin and its Margin* (eds Sutton, G. H. *et al.*) **19** (American Geophysical Union, Washington DC, 1976).
19. Hales, A. L., Hellsley, C. E. & Nation, J. B. *J. geophys. Res.* **75**, 7362–7381 (1970).
20. Leeds, A. R., Knopoff, L. & Kausel, E. G. *Science* **186**, 141–143 (1974).
21. Leeds, A. R. *Phys. Earth planet. Inter.* **11**, 61–64 (1975).
22. MacGregor, I. D. in *The Mantle Sample: Inclusions in Kimberlites and Other Volcanics* (eds Boyd, F. R. & Meyer, H. O. A.) (American Geophysical Union, Washington DC, 1979).
23. Akaogi, M. & Akimoto, S. *Phys. Earth planet. Inter.* **19**, 31–51 (1979).
24. Green, D. H. & Ringwood, A. E. *Earth planet. Sci. Lett.* **3**, 151–160 (1967).
25. Ringwood, A. E. *Composition and Petrology of the Earth's Mantle* (McGraw-Hill, New York, 1975).
26. Clark, S. P. & Ringwood, A. E. *Rev. Geophys.* **2**, 35–88 (1964).
27. Ringwood, A. E. *Earth planet. Sci. Lett.* **2**, 255–263 (1967).
28. Akaogi, M. & Akimoto, S. *Phys. Earth planet. Inter.* **15**, 90–106 (1977).
29. Jordan, T. H. in *The Mantle Sample: Inclusions in Kimberlites and Other Volcanics* (eds Boyd, F. R. & Meyer, H. O. A.) (American Geophysical Union, Washington DC, 1979).
30. Anderson, D. L. *Geophys. Res. Lett.* **6**, 433–436 (1979).
31. Cann, J. R. *Phil. trans. R. Soc. A268*, 495–505 (1971).
32. Melson, W. G., Vallier, T. L., Wright, T. L., Byerly, G. & Nelen, J. in *The Geophysics of the Pacific Basin and its Margin* (eds Sutton, G. H. *et al.*) (AGU Monogr. No. 19, 1976).
33. Stern, C. R. & Wyllie, P. J. *Am. Miner.* **63**, 641–663 (1978).
34. Kanamori, H. & Mizutani, H. *Bull. Earthquake Res. Instr.* **43**, 173–194 (1965).
35. Christensen, N. I. *J. geophys. Res.* **79**, 407–412 (1974).
36. Manghiani, M. H., Ramanantoandro, R. & Clark, S. P. *J. geophys. Res.* **79**, 5427–5446 (1974).
37. Leitner, B. J., Weidner, D. J. & Liebermann, R. C. *Phys. Earth planet. Inter.* **22**, 111–121 (1980).
38. Archambeau, C. B., Flinn, E. A. & Lambert, D. G. *J. geophys. Res.* **74**, 5825–5864 (1969).
39. Helmberger, D. V. & Wiggins, R. A. *J. geophys. Res.* **76**, 3229–3245 (1971).
40. Wang, C. J. *J. geophys. Res.* **79**, 771–772 (1974).
41. Frisillo, A. L. & Barsch, G. R. *J. geophys. Res.* **77**, 6360–6384 (1972).
42. Birch, F. in *The Earth's Crust and Upper Mantle* (ed. Hart, P. J.) **13** (American Geophysical Union, Washington DC, 1969).
43. Kumazawa, M. & Anderson, O. L. *J. geophys. Res.* **74**, 5961–5972 (1969).
44. Graham, E. K. & Barsch, G. R. *J. geophys. Res.* **74**, 5949–5960 (1969).
45. Bonczar, L. J., Graham, E. K. & Wang, H. J. *J. geophys. Res.* **82**, 2529–2534 (1977).
46. Harte, B. *Phil. Trans. R. Soc. A288*, 487–500 (1978).
47. Graham, E. K. *Geophys. J. R. astr. Soc.* **20**, 285–302 (1970).
48. Ahrens, T. J. *Phys. Earth planet. Inter.* **7**, 167–186 (1973).
49. Green, D. H. & Liebermann, R. C. *Tectonophysics* **32**, 61–92 (1976).
50. Peselnick, L., Nicolas, A. & Stevenson, P. R. *J. geophys. Res.* **79**, 1175–1182 (1974).
51. Nicolas, A. & Poirier, J. P. *Crystalline Plasticity and Solid-State Flow in Metamorphic Rocks* (Wiley-Interscience, London, 1976).
52. Ave'Lailliant, H. G. & Carter, N. L. *Bull. geol. Soc. Am.* **81**, 2203–2230 (1970).
53. Nicolas, A., Boudier, F. & Boullier, A. M. *Am. J. Sci.* **273**, 853–876 (1973).
54. Hess, H. *Nature* **203**, 629–631 (1964).
55. Raitt, R. W., Shor, G. G., Francis, T. J. F. & Morris, G. B. *J. geophys. Res.* **74**, 3095–3106 (1969).
56. Babuška, V. *J. geophys. Res.* **77**, 6955–6965 (1972).
57. Boyd, F. R. *Geochim. cosmochim. Acta* **37**, 2533–2546 (1973).
58. Danchin, R. V. in *The Mantle Sample: Inclusions in Kimberlites and Other Volcanics* (eds Boyd, F. R. & Meyer, H. O. A.) (American Geophysical Union, Washington DC, 1979).
59. Goetze, C. *Geology* **3**, 172–173 (1975).
60. Chopra, P. N. & Paterson, M. S. *Tectonophysics* (in the press).
61. Muirhead, K. J., Cleary, J. R. & Finlayson, D. M. *Geophys. J. R. astr. Soc.* **48**, 509–519 (1977).
62. Denham, D., Simpson, D. W., Gregson, P. J. & Sutton, D. J. *Geophys. J. R. astr. Soc.* **28**, 225–235 (1972).
63. Simpson, D. W. thesis, Australian National Univ. (1973).
64. Finlayson, D. M., Cull, J. P. & Drummond, B. J. *J. geol. Soc. Aust.* **21**, 447–458 (1974).
65. Hirn, A. *Geophys. J. R. astr. Soc.* **49**, 49–58 (1977).
66. Crampin, S. *Geophys. J. R. astr. Soc.* **49**, 9–27 (1977).

Regulation of transcription in expressed and unexpressed mating type cassettes of yeast

Amar J. S. Klar, Jeffrey N. Strathern, James R. Broach* & James B. Hicks

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

* Department of Microbiology, SUNY at Stony Brook, Stony Brook, New York 11790

The genes that control the a, α and a/ α cell types in Saccharomyces are carried on transposable elements known as a and α cassettes which reside at three different chromosomal loci. Examination of the transcripts by R-looping and filter hybridization indicates that each cassette is capable of producing two divergent transcripts. Cassettes at the MAT locus are transcribed constitutively. Transcription of cassettes at HML and HMR is prevented by trans-acting negative regulators.

THE genes controlling mating type in *Saccharomyces* yeasts reside on movable elements, referred to as cassettes, which become activated on transposition from silent storage sites to the mating type locus (*MAT*)^{1–5}. The particular mating type gene activated in a cell determines the mating type of that cell. Cells expressing only an α mating type gene are phenotypically α cells: that is, they can mate with *a* cells but not with other α cells. Conversely, cells expressing only an *a* mating type gene are phenotypically *a* cells: they can mate with α but not with *a* cells. Cells that express both *a* and α mating type genes display a third phenotype (designated *a*/ α): they can mate with neither *a* nor α cells and, if diploid, can undergo meiosis and sporulation. All cells contain three distinct genetic loci on yeast chromosome III at which mating type genes may reside. At two of these loci, *HML* and *HMR*, the genes are normally unexpressed. Only the gene present at the *MAT* locus (either *a* or α) is expressed in wild-type cells.

We describe here observations on the transcription of mating type cassettes located at both the *MAT* locus and the *HML* and *HMR* storage sites in a variety of strains. The results of this

study have allowed us to refine current hypotheses which deal with two specific questions of mating type regulation. First, what is the mechanism by which *MATa* and *MAT α* products determine cell type? Second, what is the mechanism which allows expression of *a* and α DNA at *MAT* but keeps them unexpressed at *HML* and *HMR*?

Genetic analyses suggest that *MATa* and *MAT α* do not code for all the cell-type specific functions, but determine cell type by regulating the expression of cell-type specific genes elsewhere in the genome⁶. Several of these structural genes involved in the sexual cycle have been identified as mutations that result in *a*- or α -specific defects in mating or as mutations defective in meiosis and sporulation of *a*/ α cells. Two complementation groups in *MAT α* and one complementation group in *MATa* have been identified and their roles in the regulation of *a*-specific, α -specific and *a*/ α -specific genes defined^{7–9}. *MAT α 1* is a positive regulator of α -specific genes (*mata1* mutants are sterile). *MAT α 2* is a negative regulator of α -specific genes (*mata2* mutants express both *a*-specific and α -specific genes and hence are sterile). *MAT α 2* is also a positive regulator of *a*/ α -specific

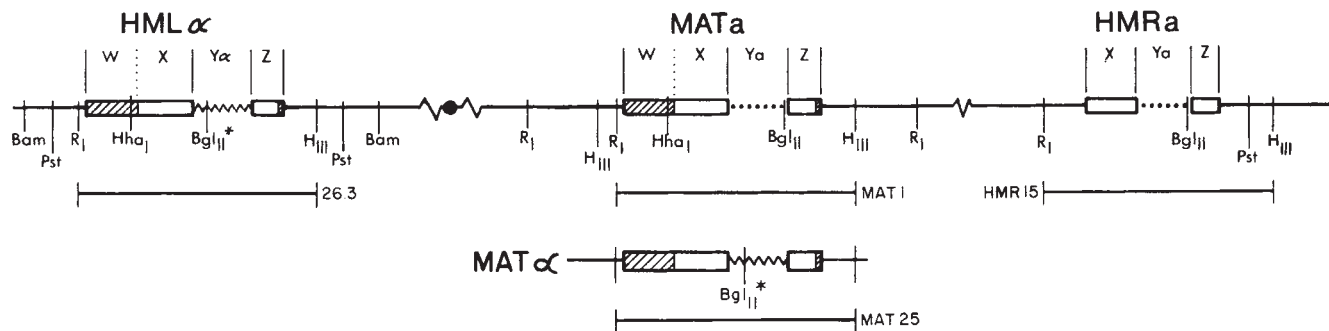


Fig. 1 Organization of the mating type cassettes on yeast chromosome III. Open boxes represent sequences common to all three loci, *MAT*, *HML* and *HMR*. Hatched regions are present at *HML* and *MAT* only. Sequences specific to *a* or α cassettes are denoted by dotted and zig-zag lines, respectively. The solid circle denotes the centromere. Detailed restriction maps are presented elsewhere⁴. Only restriction fragments used as probes in subsequent figures are labelled. The *BglII* site in the α cassettes labelled with an asterisk is presented only in strains derived from *Saccharomyces carlsbergensis*.

genes (*mat α 2*/*MAT α* diploids are sporulation defective). *MAT α* has no role in the mating ability of *a* cells (*mat α ⁻* mutants mate efficiently with α cells), but *MAT α* is required, along with *MAT α 2*, for the *a*/ α phenotype (*mat α ⁻*/*MAT α* diploids behave like α cells)¹⁰. The observations reported here contribute further to our understanding of how *MAT α* and *MAT α* regulate cell type.

There is substantial genetic and structural information available regarding the mechanism by which the mating type cassettes at the storage sites are prevented from being expressed. The two storage sites for the *a* and α cassettes, *HML* and *HMR*, are located at opposite ends of the same chromosome in which the *MAT* locus resides¹¹. Each storage site contains one copy of a mating type cassette, which can be either *a* or α ³⁻⁵. Recent structural analysis of cloned DNA molecules from *MAT* and the storage loci, *HML* and *HMR*, has shown that all three loci share two regions of sequence homology flanking a 600- or 750-base pair sequence unique to *a* or α , respectively^{3,4}. The physical structure of *HML*, *HMR* and *MAT* and their orientation on the chromosome are shown in Fig. 1. Finally, genetic analysis indicates that *HML* and *HMR* are under apparent negative control by the combined action of several distinct genes variously known as *MAR*, *SIR* and *CMT*¹²⁻¹⁴. These genes are genetically unlinked to each other or to *HML*, *HMR* or *MAT*: The *MAR1* locus has been mapped to chromosome IV (ref. 12). *MAR2* has been mapped to chromosome XII and is allelic to *CMT* (unpublished results). A mutation in either of these genes is sufficient to cause phenotypic expression of *HML* and *HMR*. These genes, therefore, operationally act as co-repressors of *HML* and *HMR*. In strains with the most common arrangement of storage genes, that is, *HML α* and *HMR α* , the *mar1-1* mutation yields a non-mating, *a*/ α -like phenotype, presumably because both α and *a* functions are being expressed¹².

Thus, the mating type cassettes encode products which regulate expression of unlinked genes involved in the mating and sporulation pathways and are themselves regulated when located in the storage sites. To examine the nature of these controls further, we have examined the transcription patterns of the *MAT α* and *MAT α* genes in *a*, α and *a*/ α cells. We have mapped these transcripts and assayed their expression in mutant strains defective in normal control functions. We have also examined transcription from the *a* and α elements residing at *HML* and *HMR* in normal strains and in strains carrying *mar⁻* mutations which result in the expression of these loci.

Identification of transcripts

The *MAT α* and *MAT α* alleles each produce two mature RNA transcripts. These transcripts were detected by electrophoretic separation of poly(A)⁺-selected RNA on agarose gels followed by transfer to diazotized filter paper¹⁵ and hybridization with ³²P-labelled *a* and α DNA cloned in bacterial plasmids. Figure 2 displays a series of RNA gel blots from normal haploid strains in which only the *MAT* locus is expressed and *mar1⁻* non-mating strains in which all three loci containing mating type cassettes

are expressed (discussed below). Tracks *a* and *c* of Fig. 2 contain RNA from normal *MAT α* and *MAT α* haploid strains probed with an *EcoRI*-*HindIII* fragment of *HML α* DNA, and tracks *e* and *g* contain the same RNAs probed with a *EcoRI*-*HindIII* fragment of *MAT α* DNA. The sizes of the labelled transcripts were determined relative to pBR322 restriction fragment markers run on the same gel. A comparison of these four tracks yields the following conclusions: (1) there are two transcripts from *MAT α* , one of 650 bases (*ax*) and one of approximately 450 bases (*ay*); (2) only the 650 base *MAT α* transcript is homologous with α DNA from *HML α* (Fig. 2, track *a*); (3) *MAT α* produces a broad band (tracks *c*, *g*) which consists of two transcripts of similar size, ~800 bases (α 1) and 750 bases (α 2). An additional band high in gel tracks *e*-*h* represents a transcript not associated with mating type but which overlaps the cloned *MAT* fragment used as a hybridization probe. The *MAT α* transcripts, which are not separated on the gel shown in Fig. 2, can be distinguished both by R-loop analysis in the electron microscope and by hybridization mapping, as described below. More detailed mapping of these transcripts is also presented below.

In addition to the normal haploid strains we have also analysed the patterns of transcripts in *mar1⁻* haploids. As described above, genetic characterization of strains harbouring the *mar1⁻* allele indicates that such strains express the storage loci, *HML* and *HMR*, in addition to the *MAT* locus¹². Hybridizations to RNA from *mar1⁻* strains of genotype *HML α MAT α MAT α* (Fig. 2, tracks *d*, *h*) and *HML α MAT α HMR α* (tracks *b*, *f*) are shown. In the tracks probed with *MAT α* DNA both characteristic *a* transcripts appear as well as at least one α transcript. This pattern of bands is identical with that obtained using RNA from normal *a*/ α diploid (see Fig. 4, track *j*). Therefore, we assume here that the state of mating type expression in non-mating *mar1⁻* cells is equivalent to that in a *a*/ α diploid. The relative intensities of the bands with tracks of RNA from *mar1⁻* strains correspond roughly to the gene dosage of *a* and α cassettes in each strain, suggesting that all three loci are being expressed at comparable levels. Comparison of *mar1⁻* and *MAR1⁺* strains indicates that the *HML* and *HMR* loci are apparently under transcriptional control through the *MAR* functions. Similar results have been obtained for mutants carrying the *mar2⁻* allele which defines a *MAR* function mapping on chromosome XII (data not shown). The tracks probed with *HML α* yield a similar result with the exception that the small *a* transcript is missing, indicating that this 450-base transcript comes from the sequences unique to the *a* cassette.

Mapping the transcripts

The positions of both α transcripts (α 1 and α 2) and the longer of the two *a* transcripts (*ax*) were determined by electron microscopy of R-loops formed by hybridization of poly(A)⁺ RNA to a *EcoRI*-*HindIII* fragment containing *MAT α* or *MAT α* DNA cloned in the bacterial plasmid pBR322. A histogram of

R-looped molecules is shown in Fig. 3. One α transcript was mapped to the X region of the *MAT* locus and the other extends from approximately the middle of the $Y\alpha$ region rightwards through Z. Both α transcripts would therefore be expected to show homology with the *MATa* gene as well as *MAT α* because the X and Z regions are common to both (see Fig. 1). One of the *MATa* transcripts (αx) was also found to map in the X region of the *MAT* locus. It seems to share the same left-hand end point as the homologous α transcript ($\alpha 1$) but is somewhat shorter than $\alpha 1$, as expected from the gel blotting experiments. Only two molecules containing the a transcript ($a y$) were found in our R-loop preparations. However, blotting results in Fig. 2 and additional studies described below clearly indicate that $a y$ is not homologous to the *MAT α* gene and must therefore lie in the $Y\alpha$ region of the *MATa* gene.

Additional mapping data were obtained by probing RNA blots with purified restriction fragments from cloned mating type genes as shown in Fig. 4. Tracks *a-f* in Fig. 4 represent a single pair of blotted tracks hybridized sequentially to three different probes. Tracks *a*, *c* and *e* contain RNA from *mar1*⁻ non-mating a/α -like strain, tracks *b*, *d* and *f* contain RNA from

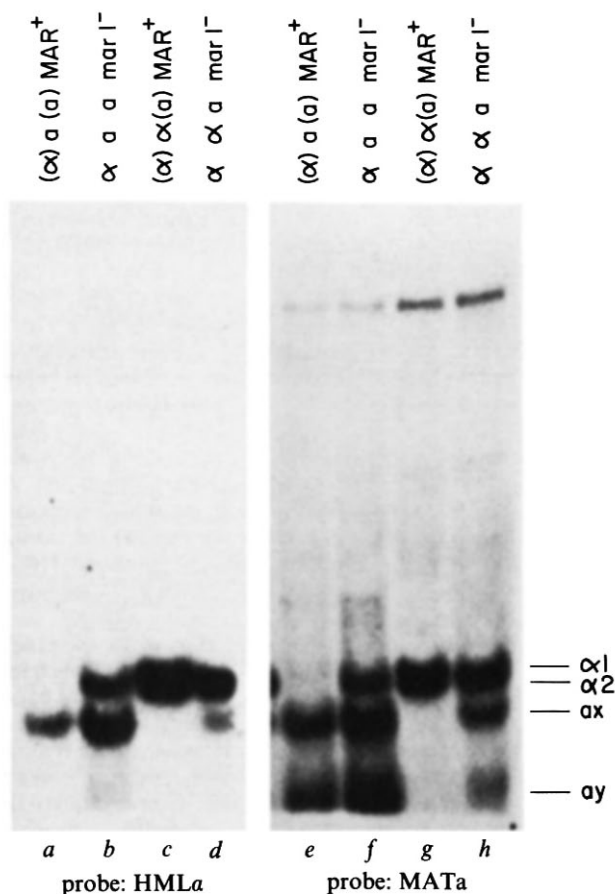


Fig. 2 Polyadenylated RNA samples (20 µg per track) were obtained from strains 2180-1A (α *MAR*⁺, tracks *a*, *e*), 2180-1B (*a* *MAR*⁺, tracks *c*, *g*), K55 (α *mar1*⁻, tracks *b*, *f*) and K54 (*a* *mar1*⁻, tracks *d*, *h*) as previously described²⁰, were fractionated on a 1.5% agarose/methylmercury hydroxide gel¹⁵ and transferred to diazobenzoyloxymethyl (DBM) paper²⁰. The immobilized RNA was hybridized with DNA, ³²P-labelled by nick-translation, spanning either *HML α* (plasmid 26.3, in Fig. 1; tracks *a-d*) or *MATa* (plasmid *MAT1*, Fig. 1; tracks *e-h*) as described previously²⁰. The washed filter was autoradiographed with Kodak XR-1 film and Dupont Lightning Plus intensifier screens for 24 h. The relevant genotypes of the strains from which the RNA was obtained are indicated above each track and indicate the mating type cassette (*a* or α) present at *HML* (bottom designation), *MAT* (middle designation) and *HMR* (top designation). Brackets around the designation indicate that the cassette at that particular locus is unexpressed, as established by genetic criteria. The absence of brackets indicates that the cassette is expressed at that particular locus. Bands corresponding to $\alpha 1$, $\alpha 2$, αx and $a y$ transcripts, identified as described in the text, are indicated to the right of the autoradiogram. Molecular weights were estimated using restriction fragments of bacterial plasmid pBR322 as standards (not shown).

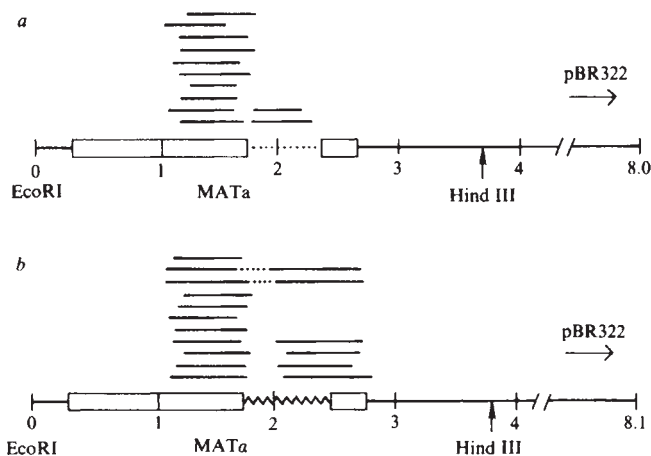


Fig. 3 Histogram of R-looped plasmid molecules containing the *MATa* gene (pBR-MAT1) hybridized with RNA from a *MATa* strain (*a*) and the *MAT α* gene (pBR-MAT25) hybridized with RNA from a *MAT α* strain (*b*). Both cloned *MAT* genes were inserted in pBR322 as *EcoRI*-*HindIII* fragments and the hybrid plasmids were digested with *EcoRI*. Dotted lines indicate individual molecules containing both R-loops, measured molecules represent approximately 50% of those observed to contain R-loops. Bacteriophage Φ X-double stranded DNA was included as a size standard. R-looping was performed according to the method of Kaback *et al.*²¹ and measurements were made on a Numonics electronic planimeter.

a normal α haploid. Tracks *a* and *b* are probed with a fragment which covers $Y\alpha$, Z and overlaps the X region of *MATa*. The probe reveals a single band in the a/α tracks, at ~750 bases, but shows a doublet at 800/750 bases in the α track. We interpret this result to mean that two transcripts are made from the *MATa* locus in strains exhibiting the α phenotype but only one in a/α strains. This interpretation was confirmed by additional hybridization studies described below. Knowing the positions of the α transcripts, we could confirm that only one of two α transcripts is present in a/α cells. This was done by testing the original filter in Fig. 4*a,b* with probes containing only X-region homology (103.4) and Z-region homology (103.5). Using the high molecular weight band associated with the right-hand end of the *MAT* probe as an internal standard it is clear (Fig. 4, tracks *c-f*) that the Z-region transcript ($\alpha 1$) is not present in the a/α strain but the X-region transcript ($\alpha 2$) is present in both α and a/α cells. The presence of two transcripts from *MATa* is consistent with the observation of two complementation groups *MAT α 1* and *MAT α 2* as described above. Further, the absence of the $\alpha 1$ transcript in a/α cells correlates with the fact that a functional *MAT α 1* allele is not required for the a/α phenotype. On the other hand, *MAT α 2* is a required function in a/α diploids. We therefore identify the message which is present only in α haploid strains ($\alpha 1$) as the transcript for the *MAT α 1* gene and the message present in both α and a/α strains ($\alpha 2$) as the transcript of *MAT α 2*. This interpretation is consistent with genetic recombination results suggesting that *MAT α 2* is proximal to *MAT α 1* (refs 7, 9) and has been confirmed by *in vitro* mutagenesis by Nasmyth *et al.* (see accompanying article¹⁶).

Figure 4 also yields information concerning the location of the a transcripts. Both αx and $a y$ transcripts are clearly present in a *HML α MAT α HMRa mar1*⁻ strain when probed with whole *MATa* DNA (Fig. 2, track *h*; see also the a/α strain in Fig. 5). However, when probed with fragment 103.8, only the smaller of the a transcripts, $a y$, is revealed in the a/α track, indicating that the 650-base transcript (αx) lies mainly to the left of the *HhaI* restriction site in the region X. Furthermore, as the small a transcript does not hybridize significantly with *HML α* DNA (Fig. 2), it must lie mainly within the $Y\alpha$ region. This interpretation is confirmed by additional hybridization of a/α and a strains with the X-region probe 103.7 (Fig. 4*g,h*) and an a strain with the Y-region probe 103.8 (Fig. 4*i*). In additional experiments (data not shown) the W region probe 103.6 was used against a and α RNA. No hybridization was detected with this

probe to any of the RNA species. Because either mating type cassette can be expressed at *HMR*¹⁷, even though the W region is not represented there^{4,5}, it was not required that those sequences be transcribed.

Direction of transcription

The direction of transcription of the mating type genes was determined by hybridization of RNA gel blots to separated strands of λ bacteriophage carrying cloned *HML* α or *HMR* α sequences followed by subsequent hybridization to wild-type λ DNA labelled with ³²P by nick translation. The orientation of the cloned genes in the λ vector was determined by heteroduplex analysis in the electron microscope. The result of these experiments is shown in Fig. 5. The tracks on the left represent parallel electrophoresis of poly(A)⁺ RNA from an α haploid strain S245c hybridized with the R and L strand, respectively, of the hybrid λ clone MAT25 (*MAT* α). The α 1 band hybridizes to the R strand of the λ clone, that is, it is transcribed from Y α towards Z, whereas α 2 hybridizes to the L strand and is thus transcribed leftward as the locus is drawn in Figs 1 and 5. The pair of tracks on the right contain poly(A)⁺ RNA from a *a/a* diploid strain probed with separated strands from λ clone *HMR*15c (*HMR* α). As described previously, this RNA preparation would be expected to contain the transcripts designated α x, α y and α 2, all of which should be detected by the *HMR*15c probe. These tracks show that α x as well as α 2 hybridizes to the L strand and is transcribed leftward in the X region of the cassette. The α y transcript hybridizes to the opposite strand and is thus transcribed to the right in region Y α . Thus, both *a* and α cassettes are transcribed divergently from within the middle of

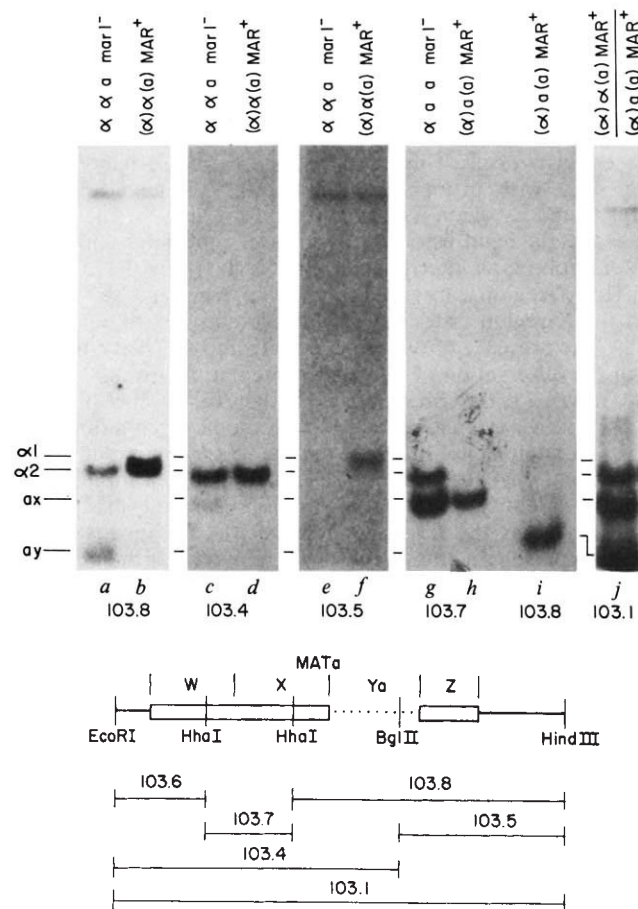
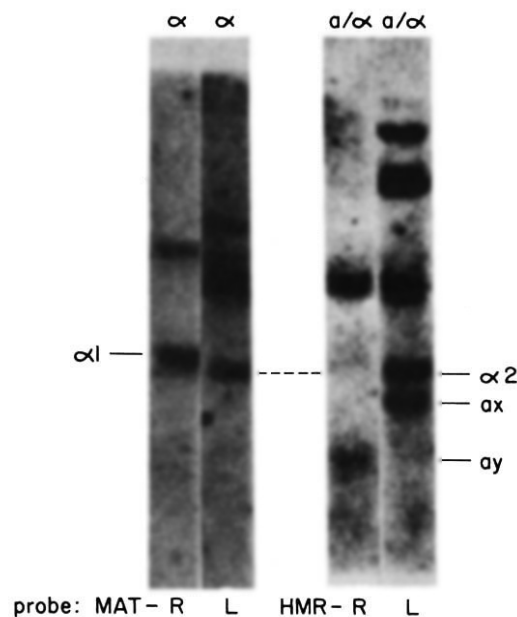


Fig. 4 Mapping the α and α transcripts by hybridization of RNA gel blots with specific probes. tracks a-f represent the same filter probed sequentially with the probes indicated. Between hybridizations the previous probe was removed by treatment with 99% formamide and 10 mM HEPES buffer, pH 7.5, at 65 °C for 1 h. Relevant genotypes of the strains used are indicated above each track as described in Fig. 2 legend. Parentheses denote silent *HML* α and *HMR* α cassettes.



MAT 25

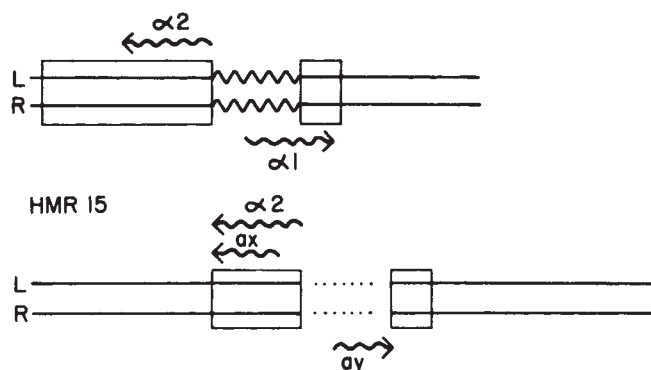


Fig. 5 Orientation of the α and α transcripts was determined using separated strands of hybrid bacteriophage λ molecules²² carrying cloned mating type cassettes as hybridization probes against RNA gel blots of the α haploid strain DC12 and the *a/a* diploid strain DC13 followed by 'sandwich' hybridization with ³²P-labelled λ DNA. Isolation of λ clones was described previously⁴. *MAT*25 carries a *Eco*RI fragment containing the *MAT* α cassette. *HMR*15 contains an *HMR* α cassette. Strand separation and hybridization have been described previously²⁰. The orientation of the cassette in each clone, and hence the designation of R and L strands, was determined relative to the short and long arms of the λ cloning vector (λ gtWES²³) by heteroduplex analysis in the electron microscope. Unlabelled bands represent non-mating type transcripts originating from flanking sequences carried on the cloned molecules.

the cassette. At least for α 1 and α y the 5' end of the transcript is inside the unique Y α or Y α sequence and therefore inside the transposed portion of the cassette. In addition to the direction of transcription, a comparison of tracks a and c in Fig. 5 provides additional evidence that the α 1 transcript is found only in cells of the α mating type and not in *a/a* diploids.

Control of α 1 transcription in *a/a* cells

The transcript mapping results described above showed that one of the two α transcripts (α 1) is not present in *a/a* (non-mating) cells. This is consistent with genetic results indicating that *MAT* α 1 is not required for expression of the *a/a* cell type but is required in α cells to turn on α -specific mating functions. We have therefore investigated whether *MAT* α 1 is turned off by the products of the *MAT* α allele (α x and/or α y) or by the combined action of *MAT* α and *MAT* α 2. Poly(A)⁺ RNA was isolated from *mata*2/*MAT* α diploids and tested for expression of the α 1 transcript by hybridization with the whole *MAT* α 1 probe (homologous to α 1, α 2, α x and α y) and a purified probe containing only the *MAT* α yz region specific to α 1. The

autoradiographs in Fig. 6 show sequential hybridizations of these two probes to the same filter containing two *mata2*⁻/*MATa* diploids, a normal *MATa*/*MATa* diploid and a *MATa* haploid strain. The $\alpha 1$ and $\alpha 2$ bands are not resolved, but the positive signal at the $\alpha 1/\alpha 2$ position in the mutant diploids when probed with *MATa*YZ indicates that the $\alpha 1$ transcript is present. As expected, no signal is seen at that position in the *MATa*/*MATa* diploid. Thus, it is clear that both *MATa2* and the product(s) of the *MATa* gene are required for turn-off of *MATa*1. The possible mechanisms of *MATa2* and *MATa* action are described further below.

Control of cell type

The results presented here, combined with genetic results described elsewhere, can be used to generate a circuit diagram for the control of cell type in *Saccharomyces*. Figure 7 shows the inferred regulatory pathways in α strains, *a* strains and *a/a* strains. In the α strain shown in Fig. 7a both *MATa*1 and *MATa*2 have active regulatory roles. Because *MATa*1 is necessary for efficient mating but not for sporulation it is proposed to be a positive regulator of α -specific functions. *MATa*2, on the other hand, is proposed to act as a negative regulator of α -specific functions. A *mata2*⁻ mutant is presumed to be defective in mating as both α and *a* mating functions are turned on.

In the mating type *a* strain shown in Fig. 7b, no control functions are assigned to the products of the *MATa* allele. This is because no recessive mutations at the *MATa* locus have been shown to cause defects in *a* mating ability. We presume, therefore, that the *a*-specific genes are constitutive in the absence of *MATa*2, and that even though *MATa* is transcribed in both *a* and *a/a* strains, its products only exert an effect in *a/a* strains.

Regulation in a *MATa*/*MATa* diploid strain is shown in Fig. 7c. In addition to the turn on of *a/a*-specific functions by *MATa*2 and *MATa*, some control mechanism shutting off *a*- and α -specific functions is required. Several genetic arguments suggest that *MATa*2 is responsible for shutting off *a*-specific functions in *a/a* cells just as it does in α cells⁹. We show here that α -specific functions are turned off in *a/a* diploids by the combined action of *MATa*2 and *MATa* repressing the tran-

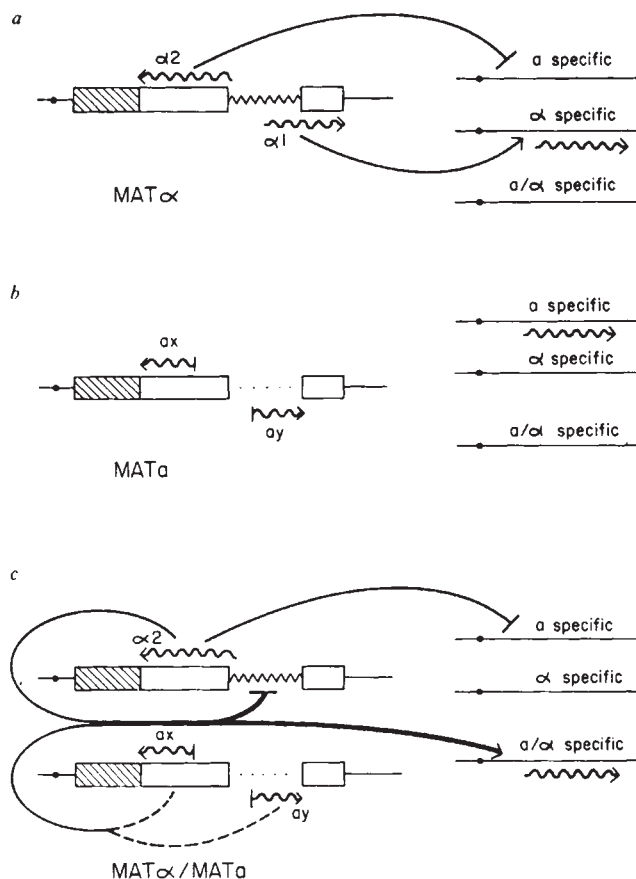


Fig. 7 Control of cell type in *Saccharomyces*. (a) α cells; (b) *a* cells; (c) *a/a* cells. Straight arrows indicate positive control. T-bars indicate negative control. Wavy arrows are the transcripts described in the text. Other symbols as in Fig. 1. The requirement of both *ax* and *ay* in control of cell type has not been determined. Genetic analyses of control of cell type are described elsewhere⁶⁻¹⁰.

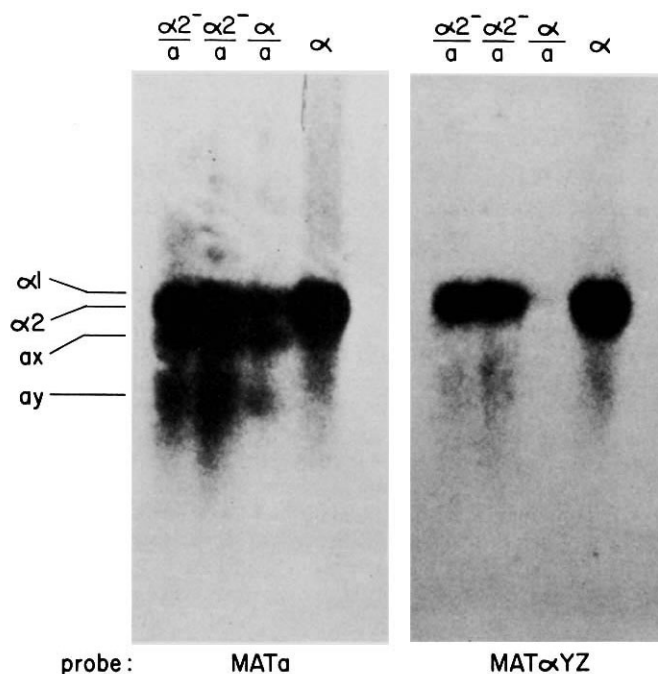


Fig. 6 Sequential hybridization of RNA from a series of strains blotted on diazotized paper²⁰ to probes containing a complete *MATa* cassette (*MATa*1, Fig. 1) and the right half of a *MATa* cassette (*MATa*YZ). Two independently isolated *mata2*⁻/*a* diploids (DC100, DC101), a normal *a/a* diploid (DC13) and an α haploid (DC12) were used. The *MATa* YZ probe was made by cutting a cloned α cassette from strain CB11 at the *Bgl*II site in the Y region⁴ yielding a probe specific for the *mata*1 transcript.

scription of the positive regulator of α genes, *MATa*1. This analysis does not determine whether it is *ax* or *ay* (or both) that are required for the *a/a* cell type. *In vitro* mutagenesis of cloned *MATa*-containing plasmids by Nasmyth *et al.* (see accompanying article¹⁶) indicates that only *ay* is necessary for this interaction. No function has been established for *ax*. We therefore propose that an interaction occurs between the *MATa*2 and the *MATa* product(s) which changes their regulatory specificities. This interaction creates a new regulatory molecule which serves to turn off *MATa*1 and also to turn on the *a/a*-specific genes whose functions are required for meiosis and sporulation. Of course, it is also possible that *MATa*2 and the products of *MATa* do not interact with each other but must act at independent sites on the DNA so as to regulate $\alpha 1$ and the *a/a* specific genes.

Control of the silent genes

In addition to the control of cell type itself the transcription mapping presented here provides insight into the mechanism by which the storage cassettes, *HML* and *HMR*, are kept silent. There is much genetic evidence indicating that *HML* and *HMR* are under negative control and the results presented here show that control is apparently at the level of transcription. At least four distinct genetic loci, known as *MAR* or *SIR*, have been identified which are required to prevent expression of *HML* and *HMR*. In both *mar1*⁻ and *mar2*⁻ mutants mature transcripts are produced at *HML* and *HMR* and these transcripts seem to be identical to their mature counterparts from *MAT*. These results are particularly surprising considering the 5' end of at least one α transcript ($\alpha 1$) and one *a* transcript (*ay*) map within the region of the cassette which must be transposed during mating type switching.

How, then, can that region of the cassette be silent at one chromosomal locus and active at another? On one hand, it is possible that the 5' end of the observed transcripts do not reflect the actual transcription initiation site. The transcripts could start near the ends of the cassettes and proceed in opposite directions, crossing each other to reach the regions maintained in the mature transcript. In this model the transcription initiation sites could be in or near regions not shared by *MAT* and the storage loci and hence subject to different control. On the other hand, transcriptional initiation may occur near the 5' ends of the mature transcripts. In this case, differential regulation of the cassettes could be effected by differential accessibility of the promoter site depending on whether the cassette is located at *MAT* or at *HML* or *HMR*. The accessibility of the promoter would be established by the interaction of sites outside the regions of homology with the *MAR* and *SIR* products and could reflect differential chromatin structure¹⁸, phasing of nucleosomes¹⁹ or differential methylation. The structure of certain intrachromosomal rearrangements⁴ suggests that the sites of action of the negative regulators lie to the left of the W-X regions at *HML* and *HMR*. Deletion of these sequences results in the expression of the normally silent *HML* and *HMR* cassettes.

Regardless of the particular mechanistic model, it is clear that mating type expression in yeast is an example of a genetic positional effect; that is, the expression of a DNA sequence depends on its location in the genome. The availability of mutants which allow expression of the silent cassettes and the existence of cloned DNA from *MAT*, *HML* and *HMR* should permit a detailed analysis of the molecular basis of this positional effect.

We thank David Kaback for assistance with the R-looping analysis, Ira Herskowitz for comments on the manuscript, Jean McIndoo, Carolyn McGill, Victoria Guarascio and Sajida Ismail for technical assistance, Louisa Dalessandro for preparation of the manuscript and Mike Ockler for the figures. This research was supported by NIH research grant GM25678-03 to A.K., GM25634-03 to J.H. and J.S. and GM24226 to J.B.

Received 8 September; accepted 14 November 1980.

1. Hicks, J. B., Strathern, J. & Herskowitz, I. in *DNA Insertion Elements, Plasmids and Episomes* (eds Bukhari, A., Shapiro, J. & Adhya, S.) 475-462 (Cold Spring Harbor, New York, 1977).
2. Harashima, S., Nogi, Y. & Oshima, Y. *Genetics* **77**, 639-650 (1974).
3. Hicks, J., Strathern, J. N. & Klar, A. J. S. *Nature* **282**, 478-482 (1979).
4. Strathern, J., Spatola, E., McGill, C. & Hicks, J. B. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2839-2843 (1980).
5. Nasmyth, K. A. & Tatchell, K. *Cell* **19**, 753-764 (1980).
6. McKay, V. & Manney, T. R. *Genetics* **76**, 273-288 (1974).
7. Strathern, J. N. thesis, Univ. Oregon (1977).
8. Hicks, J. B. thesis, Univ. Oregon (1975).
9. Strathern, J., Hicks, J. & Herskowitz, I. *J. molec. Biol.* (in the press).
10. Kassir, Y. & Simchen, G. *Genetics* **82**, 187-206 (1976).
11. Harashima, S. & Oshima, Y. *Genetics* **84**, 437-451 (1976).
12. Klar, A. J. S., Fogel, S. & Macleod, K. *Genetics* **93**, 37-50 (1979).
13. Rine, J., Strathern, J. N., Hicks, J. B. & Herskowitz, I. *Genetics* **93**, 877-901 (1979).
14. Haber, J. E. & George, J. P. *Genetics* **93**, 13-32 (1979).
15. Alwine, J. C., Kemp, D. J. & Stark, G. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5350-5354 (1977).
16. Nasmyth, K. A., Tatchell, K., Hall, B. D., Astell, C. & Smith, M. *Nature* **289**, 244-250 (1981).
17. Klar, A. J. S., McIndoo, J., Hicks, J. B. & Strathern, J. N. *Genetics* (in the press).
18. Wu, C., Bingham, P. M., Livak, K. J., Holmgren, R. & Elgin, S. *Cell* **16**, 797-806 (1979).
19. Gottesfeld, J. & Bloomer, L. S. *Cell* **21**, 751-760 (1980).
20. Broach, J. R., Atkins, J. F., McGill, G. & Chow, L. *Cell* **16**, 827-839 (1979).
21. Kaback, D. B., Angerer, L. M. & Davidson, N. *Nucleic Acids Res.* **6**, 2499-2517 (1979).
22. Szybalski, W., Kubinski, H., Hradečna, Z. & Summers, W. C. *Meth. Enzym.* **21**, 383-413 (1971).
23. Leder, P., Tiemeier, D. & Enquist, L. *Science* **196**, 175-177.

A position effect in the control of transcription at yeast mating type loci

Kim A. Nasmyth*, Kelly Tatchell*, Benjamin D. Hall*, Caroline Astell† & Michael Smith†

* Department of Genetics, SK-50, University of Washington, Seattle, Washington 98195

† Department of Biochemistry, Faculty of Medicine, University of British Columbia, 2075 Westbrook Place, Vancouver, British Columbia, Canada V6T 1W5

The two mating type loci MATa and MATα each produce two mRNAs that are transcribed in opposite and diverging directions from central promoters. Silent copies of MATa (HMRa) and MATα (HMLα) contain identical DNA sequences throughout the transcribed region, yet are not transcribed. It is concluded that sequences to the left of HMRa (and probably HMLα) must somehow affect transcription initiated at the centre of each locus 700 to 1,400 base pairs away. A possible mechanism for this position effect is discussed.

THE mating type of a yeast cell, *a* or *α*, is determined by a single locus on chromosome III called *MAT* (ref. 1) which may contain one of two types of DNA sequence^{2,3}. In heterothallic cells, *MATa* and *MATα*, are stable mendelian 'alleles' which when hemizygous or homozygous (as in *a* or *α* haploid cells or *a/a* or *α/α* diploid cells) confer an ability to mate with cells of the opposite mating type and when heterozygous (as in *a/α* diploid cells) turn off mating functions and allow the cell to sporulate¹. However, in cells containing the homothallism gene (*HO*), *MATa* and *MATα* are interconvertible, switching mating type at almost every cell division until conjugation leads to the formation of a stable *a/α* diploid whereupon the *MAT* alleles are stable^{4,5}.

In addition to the *HO* gene, an *a* to *α* switch requires another locus, *HMLα*, located on the left arm of chromosome III. Similarly, an *α* to *a* interconversion requires *HMRa*, located at the far end of the right arm of chromosome III^{6,7}. Genetic⁸ and physical^{2,3} analyses of the *MAT* loci have revealed that interconversion occurs by a replacement of information present at *MAT* with a copy of information of the opposite mating type

present at one of the *HM* loci. In other words, *HMLα* is a silent or unexpressed copy of *MATα*, and *HMRa* is a silent copy of *MATa*. The physical structures and chromosomal location of *MATa* or *MATα*, *HMLα* and *HMRa* are depicted in Fig. 1. At the centre of each locus lies a sequence specific to the mating type, either a 650-base pair *a* specific (*Ya*) or 750-base pair *α* specific (*Yα*) sequence. In most strains *HML* contains *Yα* and *HMR* contains *Ya*. The sequences flanking *Y* are found at both silent and active loci—a 700-base pair region to the left of *Y* (called *X*) and a 230-base pair sequence to its right (called *Z1*). Two further blocks of DNA are found at both *MAT* and *HML*—700 base pairs adjoining the left end of *X* (called *W*) and 90 base pairs adjoining the right end of *Z1* (called *Z2*). *W* and *Z2* are found at *MAT* and *HML* but not at *HMR*, irrespective of whether *a* or *α* information is present.

MAT seems to determine cell type by the control of unlinked *a* or *α* specific genes rather than by direct expression of all structural information required for mating⁹. *MATα* performs this function through the action of at least two genes, *α1* and *α2* (ref. 9). The *MATα1* gene product is necessary for the expres-

Table 1 Relative level of *MAT* transcripts in different strains

Genotype		RNA			
		<i>a1</i>	<i>a2</i>	$\alpha 1$	$\alpha 2$
<i>HMLα MATα</i>	<i>HMRα</i>	++	++	—	—
<i>HMLα MATα</i>	<i>HMRα</i>	—	—	++	++
<i>HMLα MATα/MATα</i>	<i>HMRα</i>	++	++	—	+
<i>HMLα MATα/mata2</i>	<i>HMRα</i>	++	++	++	++
<i>HMLα mata1/MATα</i>	<i>HMRα</i>	++	++	++	++
<i>HMLα MATα SIR1</i>	<i>HMRα</i>	ND	++	±	±
<i>HMLα MATα SIR3 or 4</i>	<i>HMRα</i>	ND	++	—	—
23°C					
<i>HMLα MATα SIR3 or 4</i>	<i>HMRα</i>	ND	++	—	+
34°C					
<i>HMLα MATα/MATα</i>	<i>HMRα</i>	—or±	—or±	++	++
<i>SAD1/SAD1</i>					
<i>HMLα MATα/a-lethal (HΔ)</i>	<i>HMRα</i>	++	++	—	+

The relative levels of each *MAT* transcript in different strains are shown. ++ signifies the highest level seen for a given transcript, + signifies a significant or 3- to 10-fold reduction, ± signifies a 10- to 100-fold reduction and — signifies our inability to detect it. No correspondence is intended for comparisons between transcripts; for these, see the text. Most of the data summarized here can be found by examining Fig. 3. Sometimes ± is written when no such transcript is visible in Fig. 3. This is due to inadequate reproduction of the latter. The Hawthorne deletion removes all DNA on chromosome III between *MAT* and *HMR*, due to an intrachromosomal recombination between these two loci³. The *SAD1* mutation is also due to a recombination between *MAT* and *HMR*, but is an interchromosomal recombination that results in the duplication of all DNA on chromosome III between *MAT* and *HMR* (J. Hicks, personal communication). ND, not determined.

sion of genes unlinked to *MAT* that specify an α mating type. *MAT α 2*, on the other hand, is necessary for repressing α specific genes which are otherwise expressed constitutively and would therefore antagonize the expression of an α mating type. This model⁹ for the determination of mating type implies that *MAT α* plays no part in the specification of an α mating type as the genes necessary are constitutively expressed in the absence of the $\alpha 2$ gene. A gene at *MAT α 1* is, however, necessary for specifying, in conjunction with the $\alpha 2$ gene, the non-mating a/α diploid cell type and therefore the ability to sporulate¹⁰.

The mating type genes, although also present at the silent loci *HMR* and *HML*, are normally exclusively expressed at *MAT*. Recessive mutations at a number of unlinked loci (*SIR1*, 2, 3 and 4)^{11,12} allow the silent copies to be expressed, suggesting that *HML* and *HMR* are under negative control of the *SIR* gene products.

Our aim here is to define the process whereby ty genes at *HMR α* and *HML α* are activated on transposition to *MAT*. We have therefore focused on the location and control of transcripts made from mating type loci. Our results reveal that *MAT α* and *MAT α* each produce two transcripts ($a1$ and $a2$, $\alpha 1$ and $\alpha 2$) neither of which is made at their corresponding silent copies *HMR α* and *HML α* , even though all transcript sequences and their immediate surroundings are present there. More precisely, the sequences flanking the left-hand end of each silent locus is

apparently responsible, in conjunction with *SIR* gene products, for preventing the initiation of transcription within the Y regions at the centre of each locus. This situation is reminiscent of position effects in *Drosophila*¹³ and similar phenomena recently discovered at the haemoglobin locus in humans¹⁴.

In addition to defining the control of transcription at the silent (*HM*) loci, our results have revealed that *MAT* controls its own transcription; *MAT α 1* and *MAT α 2* together repress the transcription of *MAT α 1* in a/α diploids, thereby ensuring their non-mating phenotype.

MAT transcripts

We have mapped RNA transcripts in the region of *MAT* by two different methods, R-looping¹⁵ and *S*₁ nuclease protection¹⁶. We have used the former to estimate the number and approximate position of transcripts and the latter to discover their direction of transcription and the position of their termini at a nucleotide level.

R-looping

The details of our R-looping are described in Fig. 2 and its legend. Figure 2A shows typical examples of R-loops formed between linearized *MAT α* or *MAT α* plasmid DNA and poly(A)⁺RNA from a or α cells. Figure 2B shows our conclusions, which can be summarized as follows. First, a cells contain two distinct RNA species homologous to *MAT α* in roughly equal abundance. One, which corresponds to the $a1$ gene¹⁷, is about 450 base pairs and is transcribed from the *Y α* region with its (centromere proximal) left-hand terminus about 100 base pairs to the right of the X-*Y α* boundary. The other transcript, which we call $a2$, is about 600 base pairs long and is found within the X region with its right-hand (centromere distal) end near the X-*Y α* boundary. Neither a transcript seems to have an intervening sequence sufficiently large to be detected by electron microscopy. Second, α cells also contain two distinct RNA species homologous to *MAT α* . One, which corresponds to the $\alpha 1$ gene¹⁷, is about 700 base pairs long and is transcribed from a region extending from the middle of the *Y α* to the end of Z2. The other, which corresponds to the $\alpha 2$ gene (ref. 17), is about 720 base pairs long and is transcribed from the X region with its right-hand end just within the *Y α* region. Neither of the α transcripts seem to have intervening sequences.

*S*₁ mapping

Figure 3 shows the strategy used to map the exact 5' and 3' termini of all four *MAT* transcripts. We have used the extent of protection from *S*₁ nuclease, by RNA of single-stranded end-labelled restriction fragments, as a measure of the distance from a labelled restriction site to an RNA terminus. The details of this analysis are described in Fig. 3 and its legend (Table 2 shows the genotype of the strains used in Fig. 3). In the text, therefore, we will confine ourselves to its conclusions.

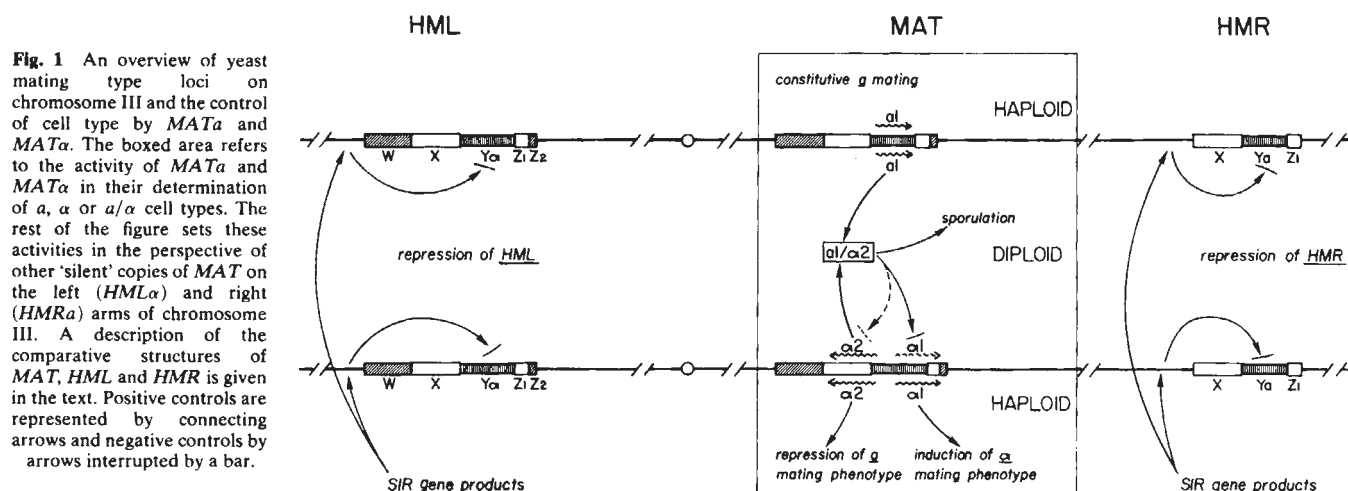


Fig. 1 An overview of yeast mating type loci on chromosome III and the control of cell type by *MAT* and *MAT α* . The boxed area refers to the activity of *MAT α* and *MAT α* in their determination of α , α or a/α cell types. The rest of the figure sets these activities in the perspective of other 'silent' copies of *MAT* on the left (*HML α*) and right (*HMR α*) arms of chromosome III. A description of the comparative structures of *MAT*, *HML* and *HMR* is given in the text. Positive controls are represented by connecting arrows and negative controls by arrows interrupted by a bar.

Table 2 Genotype of strains used

No.	Strain	Genotype	Source
S1	XP8-4A	<i>MATa ho his6 leu1 met1 trp5-1 gal2 can1</i>	P. Kushner
S2	XP8-10B	<i>MATa ho his6 leu1 met1 trp5-1 gal2 can1</i>	P. Kushner
S3	XP11	XP8-4A × XP8-10B	P. Kushner
S4	XR29-10C	<i>MATa ho sir1-1 ade6 arg4 leu2 trp1 RME</i>	G. Sprague
S5	2049-1-4a	<i>MATa ho ste8-59a (sir3) trp1 ade2 leu2</i>	L. H. Hartwell
S6	2050-5-1a	<i>MATa ho ste9-62c (sir4) trp1 ade2 leu2</i>	L. H. Hartwell
S7	265-7-4	<i>MATa ho hom3 ilv1</i>	L. H. Hartwell
S8	H293-4-2	<i>MATa ho trp1</i>	L. H. Hartwell
S9	K205	H293-4-2 × 9200-15a homozygosed for <i>trp1</i> by X rays	This work
S10	K210	K79 × 17-16 homozygosed for <i>ade2</i> by X rays	This work
S11	2760	<i>MATa ho gal12 GAL1 suc ma1 ura1 ade7</i> <i>mataΔ ho gal12 gal1 suc ma1 URA1 ADE7</i>	D. C. Hawthorne
S12	X999-1	<i>MATa SAD1 ho ade2 his4-12 leu2-27 LYS2 TRP1 ura3 gal2</i> <i>MATa SAD1 ho ADE2 his4-12 leu2-27 lys2 trp1 ura3 gal2</i>	A. Hopper
S13	K79	<i>MATa ho leu2 trp1</i>	This work
S14	17-16	<i>mata1 (a*) ho ade2 ura3 trp1 can1 cyh2</i>	A. Hopper
S15	G200-15a	<i>mata2-1 ho sir1-1 ade6 met1 his6 RME</i>	G. Sprague

(1) The *a1* and *a2* genes are transcribed in opposite and diverging directions. The *a1* transcript is initiated about 90 base pairs to the right of the X-Ya (see Figs 3, 4) boundary and extends rightward 510–530 base pairs through the Ya region (see Fig. 3, fragment 6). The *a2* transcript, on the other hand, is initiated about 50 base pairs to the left of the X-Ya boundary (Figs 3, 4) and extends leftwards through the X region for 600 base pairs (Fig. 3, fragment 1).

(2) The $\alpha 1$ and $\alpha 2$ genes are also transcribed in opposite and diverging directions. Both are initiated within the Ya region—the $\alpha 2$ gene at a point about 90 base pairs (Figs 3, 4) and the $\alpha 1$ gene at a point about 300 base pairs from the X-Ya boundary (Figs 3, 4). The *a2* and $\alpha 2$ transcripts have exactly the same termination point (Fig. 3, fragment 1).

(3) All genes, to a greater or lesser extent, have heterogeneous 5' and 3' termini. The 5' end of the *a2* transcript is a good example of this heterogeneity. Essentially the same spectrum of termini is found, barring a consistent 5-base pair difference, whether *S₁* or the single-strand specific exonuclease ExoVII (ref. 16) is used to degrade unprotected fragment 2 DNA (Fig. 3). This suggests that the pattern seen is a true reflection of RNA termini and not an *S₁* degradation artefact. We do not know, however, whether this heterogeneity of RNA termini is due to genuine heterogeneous initiation (or termination) or to processing or degradation.

(4) We have mapped all transcript sequences between their termini by the *S₁* protection method. This has been achieved either by mapping both 5' and 3' termini from a common site within the gene (for example, *MATa2*, Fig. 3) or by also analysing the protection by RNA of end-labelled DNA fragments internal to the gene (such as *MATa1*, Fig. 3). As a result, we have shown that none of the *MAT* transcripts has an intervening sequence between the termini defined by our analysis. This, however, does not exclude splicing of terminal sequences. To exclude the latter in the case of the 5' end of the *a2* gene, we have directly compared the results of *S₁* and ExoVII digestion and have found no discrepancy except for a consistent 5–7-base pair difference in the length of the protected fragment. ExoVII apparently cannot degrade the last 5 base pairs of a single-strand tail adjacent to duplex DNA (Fig. 3, fragment 2).

Control of MAT transcripts

We have studied two aspects of the control of *MAT* transcripts: (1) the regulation of transcription at the 'silent' copies of *MAT*, *HML* and *HMR* and (2) the autoregulation of *MAT* transcription by *MAT* itself.

MATa cells contain neither $\alpha 1$ nor $\alpha 2$ transcripts. Likewise, *MATa* cells contain neither *a1* nor *a2* RNAs. This implies that the 'silence' of *HMLa* and *HMRa* is due to a failure in transcription. Recessive mutations at any one of four loci (*SIR* 1, 2, 3 or 4) result in the expression of *HMLa* and *HMRa*^{11,12}. Figure 3 (fragment 9) and Table 1 show that, at least in the case of the $\alpha 2$ gene, this is due to a failure to repress *HMLa* transcription. The $\alpha 2$ transcript found in *MATa SIR1*, *SIR3* or

SIR4 strains has identical 5' termini to those found in *a/a* diploids (the most comparable genotype). In the case of *SIR3* and *SIR4*, we have analysed temperature-sensitive alleles of these loci and have shown that $\alpha 2$ transcription is also temperature sensitive.

SIR gene products, therefore, repress transcription at *HMLa*. It is not clear, however, how this repression is mediated. *MATa* and *HMLa* have identical DNA sequences^{18,19} from the left end of W to the right end of Z (a total of 2,500 base pairs), with the apparent promoter region in the middle of this homology. The specific repression of *HMLa* by *SIR* gene products must presumably be effected by flanking sequences outside this homology, beyond the 3' ends of both $\alpha 1$ and $\alpha 2$ transcripts. The same is presumably true for *HMRa*.

To characterize further the mode of *SIR* gene product action, we have analysed the levels of *a1* and *a2* transcription from two variants of *HMRa* which have different flanking sequences; the first is that caused by the Hawthorne deletion^{3,20} which replaces the left-hand flanking sequences with those from *MAT* but leaves the right-hand flanking sequences intact, and the second is the converse rearrangement, called *SAD1* (refs 21, 22), which replaces the right-hand flanking sequences with those from *MAT* but leaves the left-hand flanking sequences intact (J. Hicks, personal communication). Table 1 shows that the former produces the same amount of *a1* and *a2* mRNA as *MATa* whereas the latter produces much less of each transcript (<1%). This implies that sequences flanking the left end of *HMRa* are necessary for the *SIR*-mediated repression, whereas those flanking the right end are not. Further explanation of these variant *HMRa* loci is provided in Table 1 legend.

Regulation of MAT transcripts by MATa1 and MATa2

Figure 3 and Table 1 show that the spectrum of *MAT* transcripts in *MATa/MATa* diploids is not the sum of its *MATa* and *MATa* parents. Whereas the *a1* transcript is present in comparable amounts in *a* and *a/a* cells, the $\alpha 2$ transcript is reduced at least fivefold and that of $\alpha 1$ is entirely absent in *a/a* diploids. The $\alpha 2$ transcript starts at three discrete positions in haploids (Fig. 3 and ref. 18), one (<5%) 8 base pairs upstream from the AUG, a second (>90%) 12 base pairs upstream and a third (<5%) 28 base pairs upstream from the AUG. Only the first and second of these transcripts are subject to repression (about 10-fold reduction) in *a/a* diploids. The repression of $\alpha 1$ and $\alpha 2$ transcription in *a/a* diploids requires the action of both *MATa1* and *MATa2* genes (Fig. 3, Table 1).

DNA sequence of putative control regions in MATa and MATa

We have determined the sequence of *MATa* DNA between the starts of *a1* and *a2*, of *MATa* DNA between the starts of $\alpha 1$ and $\alpha 2$, and the corresponding region of *HML* (see Fig. 4). The following points are noteworthy. First, the region between *a1*

and $\alpha 2$ in $Y\alpha$ and the corresponding region in $Y\alpha$ have neither extensive sequence homology nor analogous sequence patterns; second, the sequence of the region between $\alpha 1$ and $\alpha 2$ in $MAT\alpha$ is identical to that in $HML\alpha$; third, the regions between the structural genes of both $MAT\alpha$ and $MAT\alpha$ contain symmetrical structures (boxed sequences of Fig. 4).

Control of silent copy transcription: a position effect

$MAT\alpha$ produces two transcripts that are initiated within 250 base pairs of each other within the unique α region ($Y\alpha$) and are transcribed in diverging directions. We have shown by DNA sequence analysis^{18,19} that for $\alpha 1$ and $\alpha 2$ the entire coding region of the mature transcripts and the sequences between them is virtually identical in $MAT\alpha$ and $HML\alpha$. However, $MAT\alpha$ and $HML\alpha$ differ drastically in their expression of identical MAT genes. $HML\alpha$ will produce neither $\alpha 1$ nor $\alpha 2$ mature transcript except when mutations at unlinked SIR loci are present. The sequence of $MAT\alpha$ and $HML\alpha$ does diverge

beyond the 3' ends of the transcripts, 800 base pairs to the left of the $\alpha 2$ transcript termination point and at the termination point of the $\alpha 1$ transcript. Somehow, then, sequences downstream of the 3' ends of the $\alpha 1$ and $\alpha 2$ genes are responsible for the differential repression of transcription at $MAT\alpha$ and $HML\alpha$ by SIR gene products.

A similar situation exists for $MAT\alpha$ and $HMR\alpha$, although here we have not demonstrated identity at the DNA sequence level. Comparison of the levels of $MAT\alpha$ transcripts in the Hawthorne deletion²⁰ and the $SAD1$ mutation^{21,22} (Table 1) suggests that sequences flanking the left (centromere proximal) but not the right of $HMR\alpha$ are primarily responsible for mediating the repression by SIR gene products (Fig. 1).

It is possible that the observed transcripts made from $MAT\alpha$ and $MAT\alpha$ are initiated not, as they seem, within $Y\alpha$ and $Y\alpha$, but from the sequences flanking MAT . By this hypothesis, $MAT\alpha 1$ would be processed from a long precursor initiated in flanking regions to the left of MAT , and $MAT\alpha 2$ would be processed from a precursor initiated in flanking regions to its right. One might then interpret the regions of pronounced dyad symmetry in $Y\alpha$ and $Y\alpha$ as being for RNA secondary structures involved in processing. The most serious empirical objection to this hypothesis is our observation that $HMR\alpha$ with its left-hand flanking sequences replaced by those from MAT (the Hawthorne deletion) produces MAT levels of both $\alpha 1$ and $\alpha 2$ transcript, whereas with its right-hand flanking sequences replaced by those from MAT ($SAD1$), it barely produces either transcript. In addition to this, the RNA processing hypothesis does not have a convincing explanation for the phenotype of SIR mutants. For instance, one would have to postulate that the promoters flanking MAT also existed in the nonhomologous sequences flanking $HML\alpha$ and $HMR\alpha$ but that they were specifically repressed by SIR gene products.

If we reject the processing hypothesis, we are forced to conclude that sequences flanking the left end of HMR can

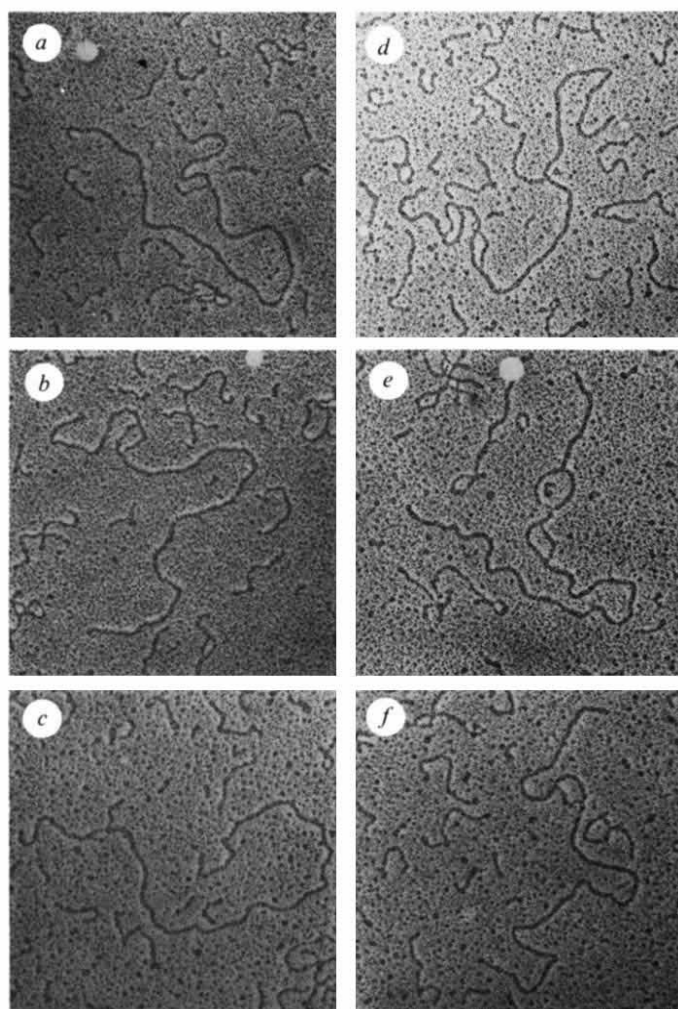


Fig. 2 R-loops formed between linearized $MAT\alpha$ or α plasmids and poly(A)⁺ RNA from α and α cells. (a) $MAT\alpha$ DNA- α RNA: shows $\alpha 1$; (b) $MAT\alpha$ DNA- α RNA: shows $\alpha 2$; (c) $MAT\alpha$ DNA- α RNA: shows $\alpha 1$ (note the tail); (d) $MAT\alpha$ DNA- α RNA: shows $\alpha 1$ and $\alpha 2$; (e) as (d); (f) $MAT\alpha$ DNA- α RNA: shows $\alpha 2$ (note the tail). All R-loops were formed between pBR322 containing a 4.3-kilobase $MAT\alpha$ or $MAT\alpha$ $HindIII$ fragment (inserted into its $HindIII$ site with the centromere distal side towards the $BamHI$ site of pBR322) and poly(A)⁺ RNA made from α (S_1) or α (S_2) cells. The procedure used was essentially that described by Kaback *et al.*²⁷. This method involves the use of cross-linked DNA which occasionally results in R-loops being interrupted by a cross-link; see, for instance, (d). Plasmid DNA was linearized by restriction with endonuclease $BamHI$, extracted once with phenol/chloroform, ethanol precipitated, cross-linked with trioxsalen (2–3 cross-links per molecule) and hybridized at 52°C for 15–18 h at 5 $\mu\text{g ml}^{-1}$ with 1 mg ml^{-1} poly(A)⁺ RNA in a total volume of 25 μl sealed in a glass capillary. After hybridization, 4 μl of deionized glyoxal (Baker) was added to the pre-cooled (to 0°C) hybridization mixture, which was then incubated at 12–14°C for 2 h. Spreading was performed directly without prior purification of RNA loops from the unhybridized RNA, by diluting an aliquot of the glyoxalated mixture with 9 vols of 0°C TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and adding 1–2 μl of the diluted R-loops to 100 μl of spreading solution (50% formamide to be spread on a 20% formamide hypophase). Spreads, their preparation for electron microscopy, their viewing and photography were performed as previously described². A quantitative analysis of the R-loops seen is shown in the lower half of the figure. We have seen R-loops within most regions of the MAT $HindIII$ fragment, but have only been able to characterize properly transcripts from its central MAT region, the others being too rare and having insufficiently precise end points to be characterized by this technique (see later S_1 mapping). $MAT\alpha$ and $MAT\alpha$ both produce two discrete transcripts that are apparent in the histograms beneath the R-loop pictures. The apparent difference between $MAT\alpha$ and $MAT\alpha$ in the occurrence of flanking transcripts is probably due to the MAT specific transcripts from the former being at a lower level (of detection, possibly) than those from the latter. Underneath the histogram is a map of MAT with the position of MAT transcripts placed on it. These positions were estimated by averaging the end points of the molecules observed. The $MAT\alpha$ transcripts both overlap homologous (X or Z) and unique ($Y\alpha$) regions. Thus, one might predict the occurrence of different types of R-loop if α RNA was hybridized instead to $MAT\alpha$ DNA. One should find in the case of $\alpha 2$ a slightly shorter R-loop with a short RNA tail and in the case of $\alpha 1$ a very much shorter R-loop with a long RNA tail. We have consistently found the latter structure in the predicted position but have only found individual examples of the former, (see (c) and (f), respectively).

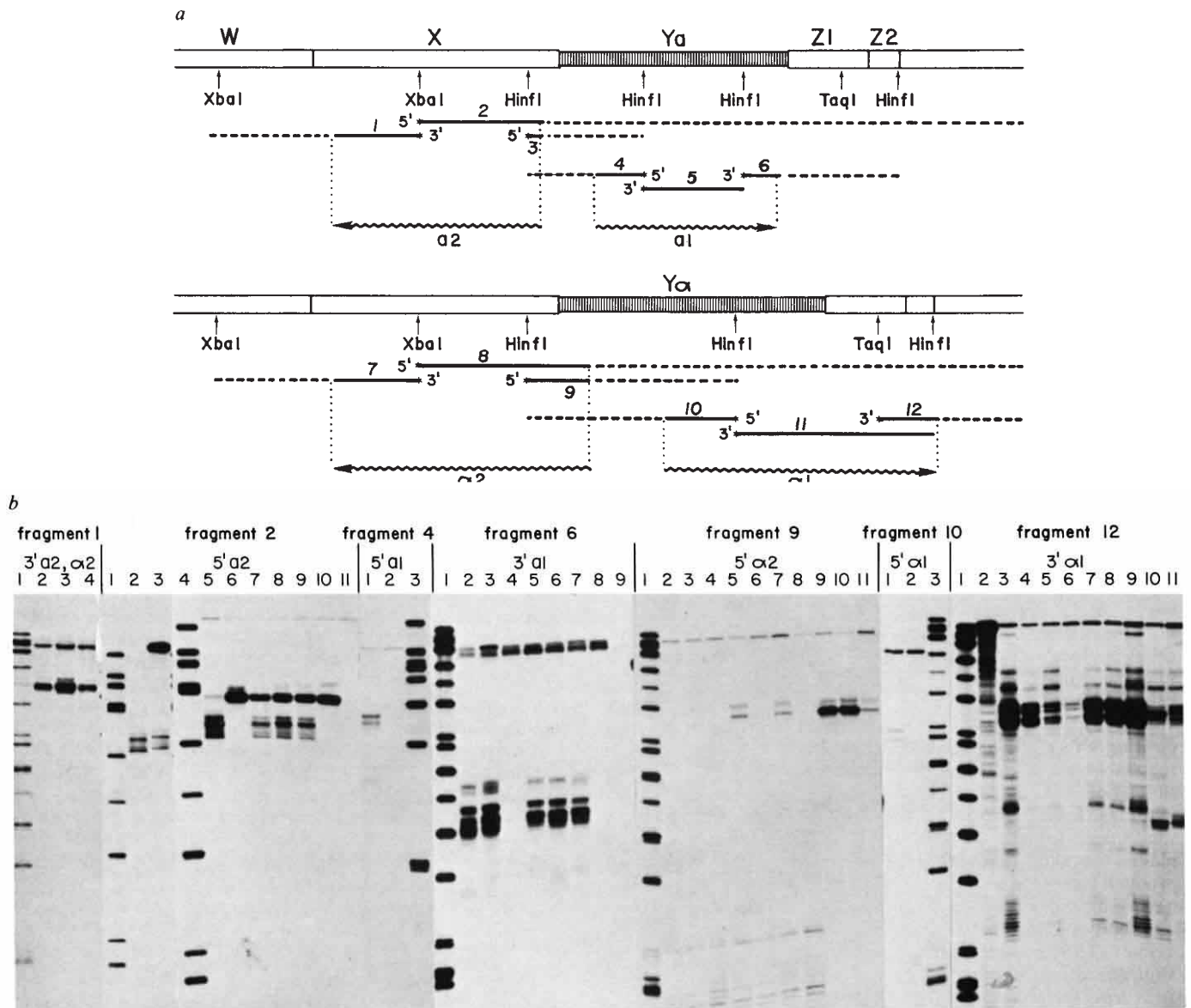


Fig. 3 S_1 transcription mapping. The upper part of the figure shows the strategy by which we have mapped the exact 5' and 3' termini of all four *MAT* transcripts. By hybridizing total poly(A) RNA to end-labelled separated strands of restriction fragments, then measuring the lengths of the 32 P-DNA strands remaining after nuclease digestion, we have measured the distance from each RNA terminus to a physically mapped restriction site. The single-stranded, end-labelled fragments used as probes are given numbers 1–12. The *Xba*I, *Hinf*I and *Taq*I fragments were derived from wild-type *MATa* or *MATa* DNA (*Hinf*III fragments) cloned in pBR322. Fragment 2 was derived from an *in vitro* manufactured mutant with a *Xho*I site within *Ya* (*mata*23, ref. 17). 5' indicates an end labelled by polynucleotide kinase and 3' indicates an end labelled with Klenow DNA polymerase. Solid lines indicate stretches of DNA protected from S_1 nuclease by RNA whereas dashed lines indicate sensitivity. Apart from the four major transcripts presented here, we have evidence for at least three more transcripts within the *MAT* region (W–Z of fig. 2). One of these, made at least in α cells (see R-looping), covers part of the W region. Another (called *MAT*3), made from both *MATa* and *MATa* in α/α cells, starts some point before the *Taq*I site of Z1 and ends at the same points as the *MATa* 1 transcript. S_1 protection experiments comparing α/α cells with a lethal (*Hawthorne deletion*)/ α cells²⁰ show that half of these *MAT*3 transcripts cease to have any homology to *MATa* from a point 88 base pairs from the *Taq*I site. This implies that the Z1–Z2 boundary occurs at this point. A third minor transcript, made at least in α cells, starts somewhere to the right of the *Taq*I site to the right of the Z1 *Taq*I site, and ends somewhere near or close to the left of the latter. We do not know the biological significance of these minor transcripts. Only those restriction sites used for S_1 mapping are shown. The lower half of the figure presents the autoradiograms of end-labelled fragments. The markers are either Φ X174 or pBR322 cut with *Hinf*I and end-labelled with Klenow DNA polymerase. The numbers in parentheses after the *MAT* genotypes refer to the strain number of Table 2. Unless otherwise stated, all digestions were performed with S_1 nuclease and cells were grown at 30°C in YEED (1). **Fragment 1 (or 7):** The 3' end of *MATa*2 and *MATa*2. Protection of fragment 1 or 7 by RNA at 1 mg ml⁻¹. (1) Φ X174; (2) *MATa* (S1); (3) *MATa* (S2); (4) *MATa*/MATa (S3). **Fragment 2:** The 5' end of *MATa*2. Protection of fragment 2 by RNA at 1 mg ml⁻¹ (lanes 2, 3) or 2.5 mg ml⁻¹ (lanes 5–10). (1) Φ X174; (2) *MATa* (S1)— S_1 nuclease; (3) *MATa* (S1)—ExoVII; (4) Φ X174; (5) *MATa* (S1); (6) *MATa* (S2); (7) *MATa*/MATa (S3); (8) *mata*1/MATa (S10); (9) *MATa*/mata2 (S9); (10) *MATa*/MATa *SAD*1/*SAD*1 (S12); (11) no RNA. The intense band(s) at 400 nucleotides in lanes 6–10 corresponds to α 2 RNA, which is also homologous to the probe up to the X–Y boundary. **Fragment 4:** The 5' end of *MATa*1. Protection by RNA at 1 mg ml⁻¹. (1) *MATa* (S1); (2) *MATa* (S2); (3) Φ X174. **Fragment 6:** The 3' end of *MATa*1. Protection by RNA at 2.5 mg ml⁻¹. (1) Φ X174; (2) *MATa* (S1); (3) *MATa* (S7); (4) *MATa* (S2); (5) *MATa*/MATa (S3); (6) *mata*1/MATa (S10); (7) *MATa*/mata2 (S9); (8) *MATa*/MATa *SAD*1/*SAD*1 (S12); (9) no RNA. **Fragment 9:** The 5' end of *MATa*2. Protection by RNA at 1 mg ml⁻¹. (1) Φ X174; (2) *MATa* (S1) grown at 23°C; (3) *MATa* (S1) 34°C; (4) *MATa* *SIR*3 (S5) 23°C; (5) *MATa* *SIR*3 (S5) 34°C; (6) *MATa* *SIR*4 (S6) 23°C; (7) *MATa* *SIR*4 (S6) 34°C; (8) *MATa* *SIR*1–1 (S4) 23°C; (9) *MATa* (S2) 23°C; (10) *MATa* (S2) 34°C; (11) *MATa*/MATa (S3) 30°C. **Fragment 10:** The 5' end of *MATa*1. Protection by RNA at 1 mg ml⁻¹. (1) *MATa* (S2); (2) *MATa*/MATa (S3); (3) pBR322. **Fragment 12:** The 3' end of *MATa*1. All lanes except lane 2 show protection of fragment 12 by RNA at 2.5 mg ml⁻¹. Lane 2 shows protection of the opposite strand to fragment 12. (1) Φ X174; (2) *MATa* (S2); (3) *MATa* (S2); (4) *MATa* (S2) 0.5 mg ml⁻¹ RNA; (5) *MATa* (S1); (6) *MATa*/MATa (S3); (7) *mata*1/MATa (S10); (8) *MATa*/mata2 (S9); (9) *MATa*/MATa *SAD*1/*SAD*1 (S12); (10) *MATa* *sir*1–1 (S4); (11) *MATa* *sir*3 (S5) grown at 34°C. Lanes 5 and 6 show bands characteristic of α 1 termini even though no transcripts with the 5' sequences of α 1 have been detected in these strains (see fragment 10). These termini probably belong to a transcript (*MAT*3?) that starts in Z and terminates at the same points as α 1 (see below). Klenow DNA polymerase and T4 polynucleotide kinase were used to label the 3' and 5' ends, respectively, of restriction fragments which were then strand separated by electrophoresis. Kinase reactions and strand separations were done as described by Maxam and Gilbert²⁸ and the Klenow polymerase I reaction and all further procedures as described by K.A.N. *et al.*¹⁸. Poly(A)⁺ RNA and single-strand end-labelled DNA fragments were annealed at 65°C followed by S_1 (or ExoVII) digestion. After digestion the samples were ethanol precipitated, redissolved in NaOH-urea loading buffer and run on 6.0% or 8.0% acrylamide sequencing gels²⁸. Gels were exposed to X-ray film plus intensifying screen at –70°C for 1–14 days.

somehow affect the initiation of transcription within *Ya*, a situation reminiscent of position effects in *Drosophila*¹³ and similar phenomena at the haemoglobin locus in humans¹⁴.

Possible mechanisms for the position effect

Hypothetical mechanisms for the position effect can be divided into two distinct types: one in which the DNA sequence is solely responsible for the effect and another in which chromosome structure is also involved.

The first type of mechanism could, for example, involve the action of an enzyme like the bacterial type I restriction endonucleases²³, which can recognize a particular sequence on a DNA molecule and then modify or cut the same molecule at a point distant from the recognition site. It is possible that the recognition site for such an enzyme (a *SIR* gene product?) exists within the left-hand flanking region of *HML α* and *HMR α* and that DNA modification (or some other interaction) subsequently occurs within *Ya* or *Y α* .

There are, of course, many variants of the second type of mechanism in which chromosome structure has an important role. These range from the very general, that *HML* and *HMR* sit within a heterochromatic region, to the very specific, that the precise composition or phasing of nucleosomes is responsible. One can, in fact, distinguish two general pathways whereby the left-hand flanking sequences of *HML* and *HMR* could influence the structure of *Ya* and *Y α* . Information from the flanking sequences could be transmitted to *Ya* or *Y α* either directly by some form of physical bridge or indirectly along the axis of the DNA molecule separating them. Higher order chromosome folding might be involved in the case of the former, whereas the precise phasing of nucleosomes might be involved in the latter.

We propose that of all the possible explanations for the position effect, the most readily tested is the notion that the sequences flanking the left end of *HML* and *HMR* are responsible, possibly in conjunction with *SIR* gene products, for initiating, from left to right, a precise phasing of nucleosomes such that promoters of transcription at *Ya* or *Y α* are either hidden or sites for repressors of transcription (*SIR* gene products) are exposed at these two loci but not at *MAT*. *MAT*, having different flanking sequences, might either be phased differently or not at all (as might be the case for a repressor model). The phasing of nucleosomes could affect the availability or activity of control sequences at *Ya* or *Y α* at two levels. At a gross level, it could be responsible for bringing sequences into juxtaposition or not; for instance, by their being wrapped around the same rather than a neighbouring core particle. At a finer level, it could also, or instead, be responsible for the exact orientation of sequences with respect to their coiling around a core particle; for instance, whether they face in or out.

Possibly relevant to the phasing hypothesis is the recent observation by Hicks (personal communication) that restriction fragments containing *HML α* and *HMR α* both contain replication origins whereas that containing *MAT* does not. If the inheritance of nucleosomes is conservative, one must postulate some means by which the naked daughter chromatid is rapidly covered with in-phase nucleosomes. This could be more readily achieved if the replication origins are also origins of phasing, as found in the case of SV40²⁵. The proximity of replication origins at the silent copies may be sufficient to effect the correct phasing. On the other hand, one or more *SIR* gene products may be involved in initiating as well as maintaining phasing.

Whatever the mechanism of the position effect on the expression of *MAT* genes, it is worth pointing out possible parallels with various phenomena associated with mating type interconversion. For instance, how does a homothallic yeast cell distinguish *MAT* from *HML* as the target of an interconversion when they are so extensively homologous? It is tempting to speculate that the same property that distinguishes their transcription may also be used to distinguish their roles as donor and recipient in an interconversion of mating type. This notion raises the question of the role of recombinant mating type loci (such as the *MAT/HMR* recombinant caused by the Hawthorne

deletion) in an interconversion; that is, whether they serve as donors or recipients. Pursuing the analogy still further, a conservative inheritance of nucleosomes at *MAT* or its silent copies may be responsible for the ability of mother cells, but not daughter cells, to undergo mating type interconversion^{4,5}.

Regulation of *MAT* transcription and the determination of cell type in yeast

Genetic analyses have identified three genes involved in the specification of cell type in yeast: $\alpha 1$, $\alpha 2$ and *a1* (refs 9, 10). The present investigation has shown that *MAT α* and *MAT α* each produce two major transcripts starting in or near their Y regions, one going leftwards and another rightwards. A series of experiments in which plasmids containing *MAT α* or *MAT α* were mutated *in vitro* and subsequently tested for *in vivo* function have shown that the left α transcript corresponds to the $\alpha 2$ gene, the right α transcript to the $\alpha 1$ gene, and the right *a* transcript to the *a1* gene¹⁷. This leaves the left (*a2*) transcript unassigned to a known complementation group. The same series of experiments implied that this transcript was, however, not essential for any of the known functions performed by plasmids carrying a wild-type *MAT α* fragment; that is, deletions within the *a2* region had no effect on the plasmid's ability to suppress α mating functions or to induce sporulation (in α/α cells). We therefore believe the $\alpha 1$, $\alpha 2$ and *a1* genes to be both necessary and sufficient for specifying all three yeast cell types: the *a* mater, the α mater and the non-mating but sporulating *a/\alpha* cell¹.

How, then, do these three genes determine cell type? Strathern *et al.*⁹ have suggested that the $\alpha 1$ gene is necessary for promoting the expression of unlinked α specific mating genes and the $\alpha 2$ gene for repressing the expression of unlinked and constitutive *a* specific mating genes. With both of these active, as in α haploids, α specific mating genes will be expressed but *a* specific mating genes not. In the absence of either, as in *a* haploids, only the constitutive *a* specific mating genes will be expressed. The presence, albeit lower, of $\alpha 2$ and complete absence of $\alpha 1$ transcripts in *a/\alpha* diploids therefore accounts for the non-mating phenotype of these cells (Fig. 1).

The repression of *MAT α* transcription that results in the complete absence of $\alpha 1$ and lowering of $\alpha 2$ transcripts in *a/\alpha* cells requires both the *a1* and $\alpha 2$ genes. The sequence between the promoters of the $\alpha 1$ and $\alpha 2$ genes contains a symmetrical region that may be the target of the hypothetical repressor of $\alpha 1$ and $\alpha 2$. No similar structure is seen near the start of the *MAT α* 1 transcript, which we have shown to be refractory to the repression by *a1* and $\alpha 2$.

The *a1* and $\alpha 2$ genes are necessary not only for suppressing mating functions in *a/\alpha* diploids but also for inducing their ability to sporulate. This function cannot be just a consequence of turning off $\alpha 1$ and turning down $\alpha 2$ because it can be expressed in circumstances in which the repression of $\alpha 1$ and $\alpha 2$ is absent²¹. It is, therefore, an independent property of the *a1/\alpha 2* state (Fig. 1). Our analysis of transcription in α/α *SAD1/SAD1* strains suggests that the induction of sporulation by *a1/\alpha 2* requires less *a1* gene product than does the repression of $\alpha 1$ and $\alpha 2$ transcription. The existence of recessive mutations (for example, *CSP*, ref. 26) that allow sporulation of *a/a* or α/α cells suggests that sporulation is normally under negative control. It is tempting to speculate that the *a1/\alpha 2* repression of $\alpha 1$ transcription and induction of sporulation are mediated by a common mechanism; that is, the transcription of *CSP* is repressed by *a1/\alpha 2* in a manner similar, though not identical, to that of $\alpha 1$.

As the amino acid sequence of all *MAT* gene products has recently been deduced from their DNA sequence^{18,19}, we may speculate on the physical mechanisms by which they control cell type. We know that some of the consequences of their action are regulation of transcription. However, we do not yet know whether they act so themselves or through intermediates. With this in mind, it is interesting that the *MAT* gene products are all basic proteins that may be capable of binding to DNA.

MAT_a

HinfI

TGGCCCTAGATAAGATCCTAATATATCCCTTAATTCAACTTCTTCTTGTGTACACTCTCTGGTAACCTAGGTAATACAGCAAATAGAAAGAGCTTTTATTTCTTGTATTTTG
 ACCGGGATCTATTCTTAGGATTATATAGGGAATTAAGTTGAAGAAGAAGACAACATGTGAGAGACCATTTGAATCCATTTATGTCGTTTATCTTTCTCGAATAATAAGAACTAAAAAC

Start a2 RNA

Start a1 RNA (M) D D I C S M A E N I

TTCCTTCGGGGAACTGTATAAACTTCCAAAAAGGAAAGTAAACAATACATCTCCTTATATCAAGAAATCAAGAGGACACATGGATGATATTTTATGATGGCGGAAACATA
 AAGAAAGCCCTTTGACATATTTGAAGGTTTTCTTTTCATTTTGTATGTAGAGGAATATAGTTCTTTTGTCTTCTGTTGACCTACTATAAAGATCATACCGCCTTTTGTAT

MAT_α, HML_α

AAGGCTTTAATGGGTATTTTATTCATTTTTCTTCTTATCTTCTTTTCTTGGCCACTTCTAAGCTGATTTCAATCTCTCTTTATATATATTTTTAAGTTCCAACATTTTATGT
 TTCCAGAAATACCCATAAAATAAGTAAAAAGAACGAATAGAGGAAAAAGAACGGGTGAAGATTGACTAAAGTTAGAGAGGAAATATATATAAAATCAAGGTTGTAATAATACA
 L D K I P I K N (M)

Start α2 RNA

TTCAAAACATTATGATGCTGGGTTTGTGTTGGGATGCAATTTATTGCTTCCCAATGTACAAAAGTACATCATATGAACAACCTTAACTCTTAACTACTTCTTTAACCTTCACTTT
 AAGTTTGTAAATCTACAGACCCAAACAAACCTTACGTTAAATTAACGAAGGTTACATCTTTTCACTGTAGTACTTTGTTGAATTTGAGAATTGATGAAGAAATTTGGAAGTCAAAA

Start α1 RNA

(M) F T S K P A F K I K N K A S K S Y R N T A V

TATGAAATGTATCAACCATATATAATACTTAATAGACGACATTACAAATATGTTTACTTGAAGCCCTGCTTTCAAAATTAAGAACAAGCATCAAAATCATACAGAAACACAGCGGTTT
 ATACTTTACATAGTTGGTATATATTGAATTATCTGCTGAAGTGTATACAAATGAAGCTTCGGACGAAAGTTTAAATCTTGTTCGTAGGTTAGTAGTCTTTGTGTCGCCAA

Fig. 4 DNA sequences around the starts of *MAT* transcripts. The DNA sequences around and between the starts of *MAT_a* and *MAT_α* transcripts are shown. The sequences were determined by the method of Maxam and Gilbert²⁸. The sequencing strategy will be described elsewhere along with a more complete sequence of the region¹⁹. In *MAT_a*, the X-Ya boundary is marked by a vertical bracket. The equivalent boundary for *MAT_α* and *HML_α* (X-Ya) is 48 base pairs beyond the left end of the sequence shown. Putative coding sequences are signified using the one-letter amino acid code. Those of the a2 transcript are not marked because there is no evidence that this transcript encodes a functional protein. Arrows mark the start points of RNAs as determined by S₁ mapping. Each transcript has two or more start points. The boxed sequences are regions that have a dyad symmetry around an axis marked with an asterisk. At each end of the α symmetrical region is a 7-base pair inverted repeat sequence that would extend the region of perfect symmetry except for 7 extra base pairs inserted between it and the left-hand end of the symmetrical region. The dashed boxes in the *MAT_a* sequence are regions of symmetry with respect to purine and pyrimidine content.

We thank Hal Weintrub for helpful discussions on nucleosome phasing, Don Hawthorne and George Sprague for supplying crucial strains, and Jane Garnett for help in compiling the paper. This investigation was supported by a grant to B.D.H. from the National Institute of Medical Sciences, a Jane Coffin Childs postdoctoral fellowship awarded to K.A.N. and a Damon Runyon-Walter Winchell Cancer fund postdoctoral fellowship to K.T.

- Hicks, J. B., Strathern, J. N. & Herskowitz, I. in *DNA Insertion Elements Plasmids and Episomes* (eds Bukhari, A. I., Shapiro, J. A. & Adhya, S. L.) 457-462 (Cold Spring Harbor Laboratory, New York, 1977).
- Strathern, J. N., Hicks, J. & Herskowitz, I. *J. molec. Biol.* (in the press).
- Kassir, Y. & Simchen, G. *Genetics* **82**, 187-202 (1976).
- Rine, J., Strathern, J., Hicks, J. & Herskowitz, I. *Genetics* **93**, 877-901 (1979).
- Klar, A. J. S., Fogel, S. & Macleod, K. *Genetics* **93**, 37-50 (1979).
- Lewis, E. B. *Adv. Genet.* **3**, 73-113 (1950).
- Van der Ploeg, L. H. T. *et al. Nature* **283**, 637-642 (1980).
- Thomas, M., White, R. L. & Davis, R. W. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2294-2298 (1976).
- Berk, A. J. & Sharp, P. A. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1274-1278 (1978).
- Tatchell, K., Nasmyth, K. A., Hall, B. D., Astell, C. & Smith, M. *Cell* (submitted).
- Nasmyth, K. A., Tatchell, K., Hall, B. D., Astell, C. & Smith, M. *Cold Spring Harb. Symp. quant. Biol.* **45** (in the press).
- Astell, C. *et al. Cell* (submitted).
- Hawthorne, D. C. *Genetics* **48**, 1727-1729 (1963).
- Hopper, A. K. & Mackay, V. *Molec. gen. Genet.* (in the press).
- Kassir, Y. & Herskowitz, I. *Molec. gen. Genet.* (in the press).
- Roberts, R. J. *CRC Crit. Rev. Biochem.* **4**, 123-164 (1976).
- Leffak, I. M., Grainger, R. & Weintrub, H. *Cell* **12**, 837-845 (1977).
- Saragosti, S., Moyne, G. & Yaniv, M. *Cell* **20**, 65-73 (1980).
- Hopper, A. K. & Hall, B. D. *Genetics* **80**, 41-59 (1975).
- Kaback, D. B., Angerer, L. M. & Davidson, N. *Nucleic Acids Res.* **6**, 2499-2517 (1979).
- Maxam, A. & Gilbert W. *Meth. Enzym.* **65**, 499-560 (1980).

Received 5 August; accepted 17 October 1980.

- Mortimer, R. K. & Hawthorne, D. C. in *The Yeasts* Vol. 1 (eds Rose, A. H. & Harrison, J. S.) 385-460 (Academic, New York 1969).
- Nasmyth, K. A. & Tatchell, K. *Cell* **19**, 753-764 (1980).
- Strathern, J. N., Spatola, E., McGill, C. & Hicks, J. B. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2839-2843 (1980).
- Hicks, J. B. & Herskowitz, I. *Genetics* **83**, 245-258 (1976).
- Strathern, J. N. & Herskowitz, I. *Cell* **17**, 371-381 (1979).
- Naumov, G. I. & Tolstorukov, I. I. *Genetika* **9**, 82-91 (1973).
- Harashima, S., Nogi, Y. & Oshima, Y. *Genetics* **77**, 639-650 (1974).

Homology and non-homology at the yeast mating type locus

George F. Sprague Jr, Jasper Rine* & Ira Herskowitz

Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene, Oregon 97403

Four mutations of the α mating type locus of Saccharomyces cerevisiae have been analysed to determine their relationship to the a mating type locus. Mat_a⁺ recombinants are produced by mat_a2⁻/MAT_a but not by mat_a1⁻/MAT_a diploids. MAT_α and MAT_a thus contain regions of homology (coding for at least part of MAT_α2) and regions of non-homology (coding for at least part of MAT_α1)—the genetic determinant for cell type is larger than the non-homologous sequence seen by DNA-DNA heteroduplexes and genetic analysis. The segment transposed in mating type interconversion includes both types of sequence.

THE mating type locus determines which of three distinct and complex cell types (a, α and a/α) is expressed by the yeast,

* Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305.

Saccharomyces cerevisiae. It has been proposed that alleles of this locus, *MAT_a* and *MAT_α*, code for different regulators controlling the expression of unlinked genes necessary for mating and sporulation¹⁻³. Several lines of evidence suggest that *MAT_a* and *MAT_α* are 'complex' alleles, that is, are genetic

alternatives coding for several different functions. First, there are at least two complementation groups in *MAT α* , $\alpha 1$ and $\alpha 2$; only one complementation group is known for *MAT α* . *MAT $\alpha 1$* and *MAT $\alpha 2$* are required for mating in haploids; *MAT $\alpha 2$* and *MAT α* are required for sporulation in diploids²⁻⁵. Second, heteroduplex analysis of cloned *MAT α* and *MAT α* DNA (as well as cloned cryptic mating type information from the *HML α* and *HMR α* loci) reveals a region of non-homology (region Y) between *a* and α information of ~750 base pairs (*Y α* for *a* and *Y α* for α). Heteroduplexes between *HML α* and *MAT α* and between *HMR α* and *MAT α* show that the non-homology region is flanked by regions of apparent homology of at least 700 base pairs on one side (the X region) and at least 200 base pairs on the other side (the Z region)^{6,7}. The physical picture of the mating type locus derived from heteroduplex analysis does not provide information on the coding regions within the mating type locus. Thus, they could be entirely within the non-homology (Y), entirely within the regions of apparent homology (X and Z) or distributed throughout X, Y and Z regions. We have made detailed studies of four mutations of the α mating type locus, two in each of the known complementation groups, to determine their genetic relationship to the *a* mating type locus. To investigate whether *mata* mutations are in regions of homology or non-homology with respect to *MAT α* , we have constructed a series of *mata*⁻/*MAT α* diploids and have analysed the meiotic products for *MAT α* ⁺ recombinants. If a mutation is in a region of *MAT α* that is non-homologous with *MAT α* , genetic rescue of the wild-type allele from *MAT α* should not be possible, but genetic rescue should be possible if the mutation is in a region that is homologous with *MAT α* . The mutations analysed here (isolated by MacKay and Manney⁸) all differ: *mata 1-5* causes a temperature-sensitive mating phenotype (G. F. S. and J. R., unpublished), *mata 1-2* is an amber mutation⁹, and *mata 2-1* and *mata 2-4* have different effects on sporulation (G. Sprague and J. Strathern, unpublished). We report here results obtained with three types of diploid: *mata 1*⁻/*MAT α* , *mata 2*⁻/*MAT α* , and *mata 1*⁻/*mata 2*⁻, which show that the wild-type alleles of *mata 2* mutations but not of *mata 1* mutations are rescued from *MAT α* .

Production of *MAT α* ⁺ segregants from *mata*⁻/*MAT α* diploids

Segregants that mate as α cells are found at the same low frequency (~5 × 10⁻⁵) among the meiotic products of two *mata 1*⁻/*MAT α* diploids carrying different *mata 1* mutations—*mata 1-5* and *mata 1-2* (strains DG1 and DG2, respectively, Table 1). Subsequent crosses show that the α mating phenotype is due to the presence of a wild-type α mating type locus in these recombinants (see Table 1 legend). Because these *MAT α* ⁺ segregants may have arisen by recombination between the *mata 1* allele and cryptic α information at the *HML α* locus rather than by recombination between *mata 1* and *MAT α* , we have constructed a *mata 1-5*/*MAT α* diploid lacking cryptic α information. Such a strain (DG3, *mata 1*/*MAT α* *HML α* /*HML α* *HMR α* /*HMR α*) does not yield *MAT α* ⁺ segregants. This result indicates that the wild-type allele of the *mata 1-5* mutation cannot be rescued from *MAT α* .

In contrast to these results, *MAT α* ⁺ segregants arise at high frequency (~2 × 10⁻³) from *mata 2*⁻/*MAT α* diploids carrying either of two different *mata 2* mutations, *mata 2-4* and *mata 2-1*. This result has been documented most thoroughly for the *mata 2-4* mutation. In particular, the production of *MAT α* ⁺ segregants from *mata 2-4*/*MAT α* diploids is not dependent on the presence of the *HML α* allele (Table 1: compare strain DG5, which is homozygous for *HML α* and *HMR α* , with strain DG4, which is *HML α* *HMR α*). Two observations indicate further that the *MAT α* ⁺ segregants are the products of recombination between *mata 2-4* and *MAT α* . First, the frequency of *MAT α* ⁺ segregants is at least 100-fold higher than the reversion frequency of the *mata 2-4* mutation (HG2, Table 1). More-

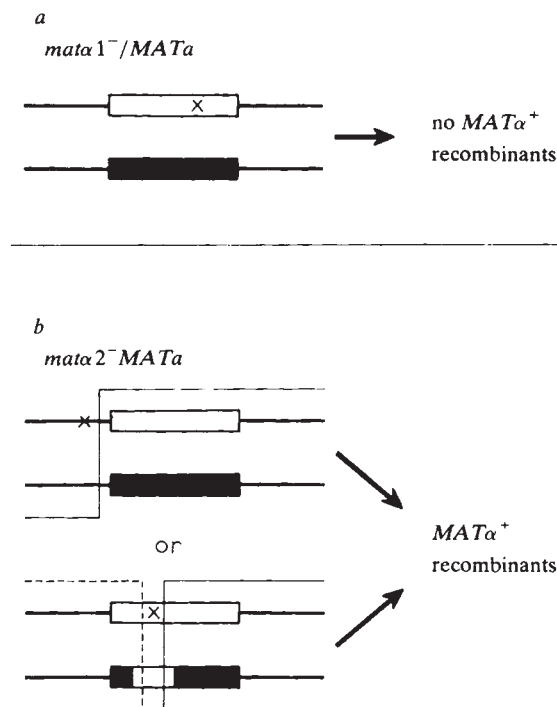


Fig. 1 Interpretation of genetic rescue experiments between *mata* mutations and *MAT α* . X, sites of *mata 1* or *MAT α* mutations. *a*, Failure to obtain *MAT α* ⁺ recombinants in crosses between *mata 1-5* and *MAT α* . Filled and open rectangles indicate non-homologous segments of *MAT α* and *mata*, respectively. *b*, Production of *MAT α* ⁺ recombinants in crosses between *MAT α* ⁻ and *MAT α* . Upper diagram: symbols as in *a*. Lower diagram: a region of homology is located between sequences that are non-homologous in *MAT α* and *mata*.

over, half of the *MAT α* ⁺ segregants are recombinant at the closely linked *CRY1* locus, which indicates that *MAT α* ⁺ has been generated by a recombination event rather than by reversion.

It is possible that the observed frequency of *MAT α* ⁺ segregants from *mata 2-4*/*MAT α* diploids is an overestimation of the true frequency, because DG4 and DG5 are defective in the *MAT $\alpha 2$* function and therefore sporulate poorly. Wild-type *MAT α* recombinants or revertants that arise in DG4 or DG5 may be enriched among the spore progeny due to efficient *MAT α* ⁺-promoted sporulation. To test this possibility, we determined the frequency of *MAT α* ⁺ segregants from a *mata 2-4*/*MAT α* diploid (DG6) homozygous for a mutation, *sir1-1*, which permits *in situ* expression of the cryptic mating type information¹⁰. This diploid sporulates well because wild-type *MAT $\alpha 2$* function is provided by α information at *HML α* . *MAT α* ⁺ segregants arise from DG6 at the same frequency as from DG4 and DG5 (Table 1). A *mata 2-1*/*MAT α* *sir1-1*/*sir1-1* diploid likewise yields *MAT α* ⁺ recombinants at high frequency (DG7, Table 1). Consequently, the frequency of *MAT α* ⁺ segregants from diploids DG4, 5, 6 and 7 seems to be a true reflection of recombination between *MAT α* and the *MAT α* ⁻ mutations and is not biased by a selection for revertants or recombinants.

To evaluate the significance of the recombination frequencies reported above, we have determined the frequency of recombination between two *mata* mutations. The frequency of recombination in *mata 1*⁻/*mata 2*⁻ diploids (~10⁻², DG8 and DG9, Table 1) is similar to that obtained from *MAT α* ⁻/*MAT α* diploids. This value for recombination across homologous mating type loci places an upper limit on the frequency of

Table 1 Frequency of α mating type clones obtained

Strain	Genotype	Frequency of α -maters	
		Clone	Frequency
DG1	<i>mata1-5</i> <i>MATa</i>	A	$\sim 4 \times 10^{-5}$ (2)
		B	$\sim 6 \times 10^{-5}$ (3)
DG2	<i>mata1-2</i> <i>MATa</i>	A	$\sim 1 \times 10^{-4}$ (2)
		B	$\sim 4 \times 10^{-5}$ (1)
DG3	<i>HMLa mata1-5 HMRa</i> <i>HMLa MATa HMRa</i>	A	$< 1.5 \times 10^{-5}$ (0)
		B	$< 1.2 \times 10^{-5}$ (0)
DG4	<i>mata2-4</i> <i>MATa</i>	A	1.6×10^{-3} (54)
		B	2×10^{-3} (60)
DG5	<i>HMLa mata2-4 HMRa</i> <i>HMLa MATa HMRa</i>	A	1.3×10^{-3} (21)
		B	1.3×10^{-3} (48)
DG6	<i>mata2-4 sir1-1</i> <i>MATa sir1-1</i>	A	3×10^{-3} (54)
		B	4×10^{-3} (54)
DG7	<i>mata2-1 sir1-1</i> <i>MATa sir1-1</i>	A	3×10^{-3} (21)
		B	2×10^{-3} (27)
DG8	<i>mata1-5</i> <i>mata2-4</i>		$\sim 4 \times 10^{-3}$ (2)
DG9	<i>mata1-5</i> <i>mata2-1</i>		9×10^{-3} (9)
HG1	<i>mata1-5</i>	A	$< 5 \times 10^{-5}$ (0)
		B	$< 3 \times 10^{-5}$ (0)
HG2	<i>HMLa mata2-4 HMRa</i>	A	$< 4 \times 10^{-5}$ (0)
		B	$< 4 \times 10^{-5}$ (0)

Strains and markers: Diploid strains were constructed by prototroph selection. Genotypes were confirmed by analysis of the segregants. HG1 and HG2 were the *mata* parents of DG1 and DG5. All diploids except DG1 were heterozygous at the *CAN1* locus, which segregates independently from *MAT* (ref. 8). DG1, 2, 3 and 5 were *cry1/CRY1*, with *cry1* coupled to *MATa* in DG2 and *CRY1* coupled to *MATa* in the others. DG3 and DG5 were shown to be homozygous for *HMLa* and *HMRa*; other strains are assumed to have the standard *HMLa HMRa* genotype. Isolation of meiotic products: Haploid segregants were isolated from two clones from each diploid (DG1, 2, 3, 4, 5, 6, 7) by random spore analysis: asci from sporulated cultures were treated with Glusulase (Endo), sonicated and plated onto medium containing cryptopleurine (for DG1) or canavanine (for the other diploids), which selects for haploids because resistance alleles *cry1* and *can1* are recessive^{8,15}. Haploid segregants were isolated from sporulated cultures of DG8 and DG9 by ascus dissection. Assay of *MATa* segregants: Spore clones with α mating type were identified by replica plating colonies onto a lawn of *a* cells and assaying mating by prototroph formation. Frequency was the number of α colonies per total number of spore clones tested. Numbers in parentheses indicate the number of α colonies observed. Subsequent crosses showed that all α colonies tested were *MATa*. Tests were performed on all α segregants from DG1, DG2, DG8 and DG9; on 3 each from DG4A, DG4B, DG5B, DG6A and DG6B, and on 19 from DG5A. Sporulation: *MATa2/MATa* strains DG1, DG2 and DG3 sporulated efficiently (60% for DG3). *mata2/MATa* strains DG4 and DG5 sporulated poorly (4% for DG5). *mata2/MATa* strains DG6 and DG7 sporulated efficiently due to homozygosity for *sir1-1* (47% sporulation for DG6). *mata1/mata2* strains DG8 and DG9 sporulated (albeit inefficiently) due to homozygosity for *rme1*, which bypasses the requirement of *MATa2* and *MATa1* for sporulation⁴.

MATa2⁺ recombinants expected from *mata2-1/MATa* or *mata2-4/MATa* diploids (for example, DG4, 5, 6, 7 above), in which *MATa2*⁺ recombinants are formed by a recombination event occurring between the *mata* mutation and the end of the *MATa* non-homology region.

Regions of homology and non-homology

Our genetic results indicate that *MATa* and *MATa* contain regions of homology as well as regions of non-homology: two sites in *MATa1* are non-homologous with *MATa*, and two sites in *MATa2* are homologous with *MATa* (Fig. 1). Studies are in progress to determine whether *MATa*⁺ recombinants can be obtained from *mata*⁻/*MATa* diploids and *mata*⁻ recombinants from *MATa*⁻/*MATa* diploids. Although the extent and position of the homology between *MATa2* and *MATa* cannot be rigorously determined from our data, the similar frequencies of *MATa*⁺ recombinants from *mata2/MATa* and *mata2/mata1* diploids lead us to favour the view that the homologous sequence is outside the non-homologous sequence (Fig. 1b, top) rather than being within it (Fig. 1b, bottom). These results therefore suggest that the genetic information determining cell type is larger than the non-homologous sequence observed in heteroduplexes between *MATa* and *MATa* (refs 6, 7). This view is reinforced by the observation that the α mating type locus codes for two transcripts, one primarily within the non-homologous region, and the other primarily outside this region^{11,12}. Our observations on homology and non-homology between *MATa* and *MATa* lead us to propose that the latter RNA codes for *MATa2* and the former for *MATa1*.

Yeast cells can change the information present at *MAT* (an *a* or α 'cassette') by replacing it with a cassette from *HML* or *HMR* (reviewed in ref. 3). This change reflects a transposition of at least the Y region information from *HML* or *HMR* to *MAT* (refs 7, 13). Is the Y region the only information that is transposed? Our findings indicate that homologous sequences are also transposed. This conclusion follows from the observations that *MATa* mutations can be switched to *MATa2*⁺ by mating type interconversion¹⁴ and, as shown here, that *MATa* mutations are outside the non-homology region. Hence, the cassette that is transposed during mating type interconversion must include not only Y information but also flanking sequence information (from X or Z).

We thank Kim Nasmyth and Jim Hicks for communicating their results before publication, Karen Sprague for comments on the manuscript, Julie Dunn for preparation of the manuscript, and David Hagen for preparation of the figure. This work has been supported by Research Career Development Award AI-00163 and USPHS research grant AI-13462 to I. H., by PHS Molecular Biology Training Grant GM-00715 (P. von Hippel, Director), and by American Cancer Society postdoctoral fellowship PF-1282 to G. S.

Received 11 August; accepted 4 November 1980.

- MacKay, V. L. & Manney, T. R. *Genetics* **76**, 273-288 (1974).
- Strathern, J. N., Hicks, J. B. & Herskowitz, I. *J. molec. Biol.* (in the press).
- Herskowitz, I. *et al.* in *The Molecular Genetics of Development* (eds Loomis, W. & Leighton, T.) 79-118 (Academic, New York, 1980).
- Kassir, A. J. & Simchen, G. *Genetics* **82**, 187-202 (1976).
- Klar, A. J. S., Fogel, S. & Radin, D. N. *Genetics* **92**, 759-776 (1979).
- Nasmyth, K. A. & Tatchell, K. *Cell* **19**, 753-764 (1980).
- Strathern, J. N., Spatola, E., McGill, C. & Hicks, J. B. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2839-2843 (1980).
- MacKay, V. L. & Manney, T. R. *Genetics* **76**, 255-271 (1974).
- Herskowitz, I., Rine, J., Sprague, G. Jr & Jensen, R. in *Mobilization and Reassembly of Genetic Information* Vol. 17 (eds Scott, W. A., Werner, R., Schultz, J. & Joseph, D. R.) 133-151 (Academic, New York, 1980).
- Rine, J., Strathern, J. N., Hicks, J. B. & Herskowitz, I. *Genetics* **93**, 877-901 (1979).
- Klar, A. J. S., Strathern, J. N., Broach, J. R. & Hicks, J. B. *Nature* **289**, 239-244 (1980).
- Nasmyth, K. A., Tatchell, K., Hall, B. D., Astell, C. & Smith, A. *Nature* **289**, 244-250 (1980).
- Hicks, J. B. & Herskowitz, I. *Genetics* **85**, 373-393 (1977).
- Strathern, J. N., Blair, L. C. & Herskowitz, I. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3425-3429 (1979).
- Skogerson, L., McLaughlin, C. S. & Wakatama, E. *J. Bact.* **116**, 818-822 (1973).

Nucleotide sequences at host-proviral junctions for mouse mammary tumour virus

John E. Majors & Harold E. Varmus

Department of Microbiology and Immunology, University of California, San Francisco, California 94143

Proviruses cloned from rat cells infected with mouse mammary tumour virus, a B-type retrovirus regulated by glucocorticoid hormones, show the structural features of transposable elements: short inverted repeats conclude long direct repeats at the ends of viral DNA, and short sequences of cellular DNA are duplicated during integration and flank each provirus. The integrative mechanism joins a precise site in viral DNA to non-homologous sites in host DNA.

INTEGRATION of a DNA copy of viral RNA into the genome of the host cell is a central event in the life cycle of retroviruses (see Fig. 1; for a review see ref. 1). The viral genome is composed of two identical subunits of single-stranded RNA, each 5–10 kilobases long. On infection, a linear duplex form of viral DNA, slightly longer than a subunit of viral RNA and bearing long terminally repeated units (LTRs)^{2–4}, is synthesized in the cytoplasm. The LTRs are composed of sequences unique to the 3' and 5' ends of viral RNA (U₃ and U₅) and a single copy of a short sequence (R) repeated at the ends of viral RNA, in a manner represented by U₃RU₅ (Fig. 1). Some of the linear unintegrated DNA is carried to the nucleus where a portion at least is converted to closed circular species⁵, mostly monomers which contain either one or two copies of the LTR sequence^{2,3,6–8}. Integration of viral DNA into the host genome can occur at a wide variety of sites in cellular DNA, possibly at random; the structure of integrated (proviral) DNA is invariably very similar, if not identical, to that of unintegrated linear DNA^{9,10}, although the immediate precursor of the provirus has not been determined. The primary product of transcription is likely to be equivalent to a subunit of genomic RNA, which can be processed to subgenomic, spliced mRNAs for expression of internal cistrons¹.

Mouse mammary tumour virus (MMTV) is a type B retrovirus with at least three genes (*gag*, *pol* and *env*) on genomic RNA subunits of eight to nine kilobases (see Fig. 1). Although its generally poor infectivity in tissue culture has impeded biochemical study, MMTV seems to replicate like other retroviruses and has the experimental advantages of causing mammary carcinomas in mice and showing dramatic regulation of viral RNA synthesis by glucocorticoid hormones in many types of infected cells (for reviews see refs 11, 12). The mechanisms of carcinogenesis and steroidal regulation by this agent are poorly understood, and an analysis of integration sites of MMTV DNA seemed likely to clarify these aspects and also the general mechanism of integration of retroviral DNA.

We used molecular cloning and DNA sequencing to analyse the structure and integration sites of three proviruses of MMTV DNA introduced into the genome of rat cells (XC cells) by infection in culture. Our analysis demonstrates the remarkable precision of the integrative mechanism and reveals additional striking similarities between the structure of proviruses and that of several transposable elements: short inverted repeats (six base pairs) within the LTRs (1.4 kilobases) terminate each provirus, and duplications of five or six base pairs of host cell DNA are generated during the integration process.

To optimize the possibility of correlating structural and functional properties of proviruses, we isolated a set of clonal rat (XC) cell lines which contain single MMTV proviruses. The lines were chosen from a larger set of clonal lines of XC cells which had been infected by about one infectious unit per cell with the C3H strain of MMTV¹³. To analyse these clones for MMTV

sequences, DNA from each clone was digested with *Eco*RI, electrophoresed through agarose, blotted on to nitrocellulose paper by the technique of Southern¹⁴ and hybridized with ³²P-MMTV cDNA. Because there is a single recognition site for *Eco*RI within the MMTV(C3H) genome, each normal provirus should yield two hybridizing fragments, the sizes of which are determined by the location of recognition sites in neighbouring cell DNA (Fig. 1b shows a restriction map of MMTV DNA).

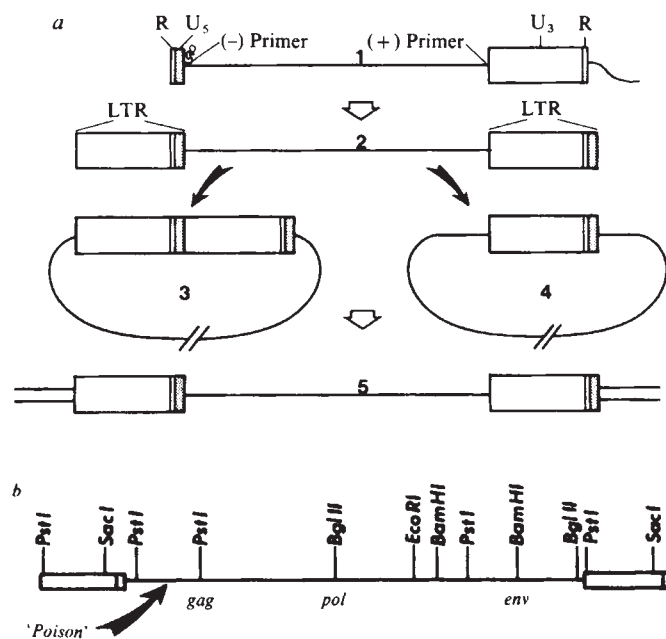


Fig. 1 a, Steps in the replication of retroviruses. The RNA subunit of the MMTV genome (1) is shown with features relevant to the present study: the site for priming of the negative (-) DNA strand by tRNA^{lys} bound near the 5' terminus^{21,22} (shown on the left); the site probably used for priming (+) strand synthesis^{2,4}, the short direct repeat (R, probably ~13 bases for MMTV) at both ends of viral RNA; and the regions unique to the 3' and 5' ends of viral RNA located between the priming sites and R (U₃, ~1,300 bases; U₅, ~130 bases). At an early step in DNA synthesis these two regions are fused via the R sequence to form the LTR U₃RU₅, which is in turn duplicated during the completion of linear duplex DNA^{2–4,6}. The linear DNA (2) is a precursor to the two circular forms which contain either two copies (3) or one copy (4) of the LTR unit^{2,3,6,7}. One of these unintegrated forms (2, 3 or 4) is presumably the precursor to the integrated (proviral) form^{9,10,23} (5), which serves as the template for synthesis of viral RNA. b, A map of restriction sites in MMTV-C3H DNA which are relevant to the present study^{6,23}. As described in the text we have been unable to clone the region marked 'poison'.

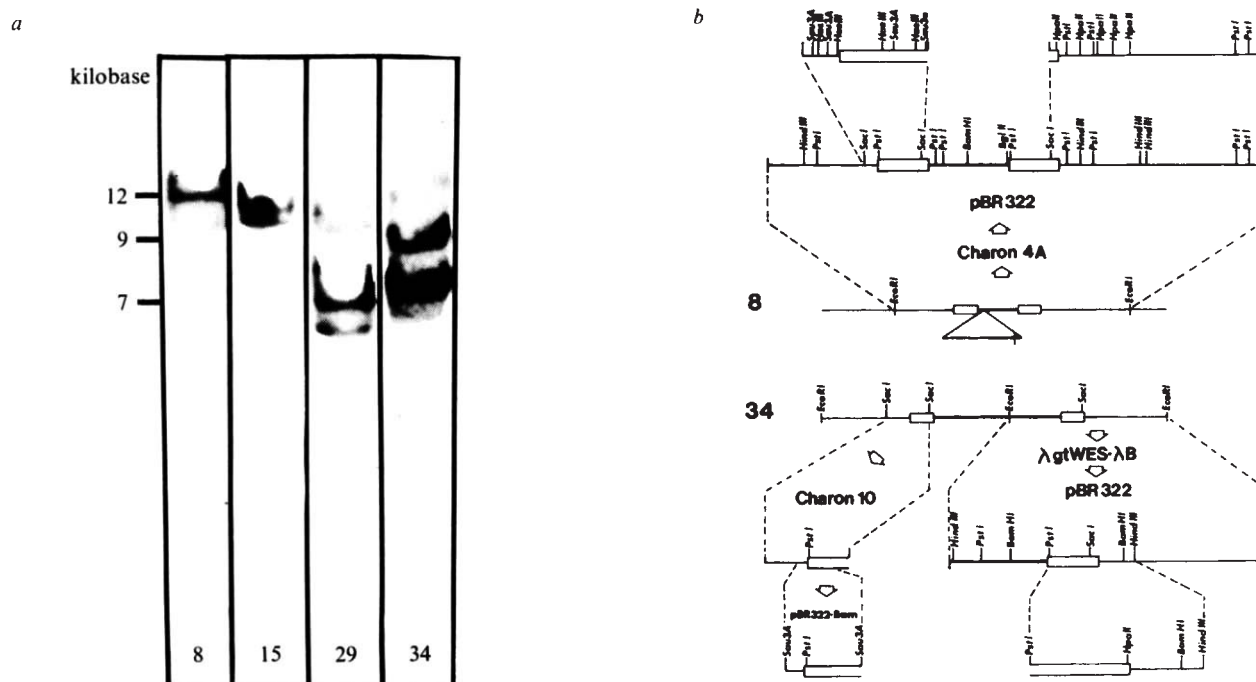


Fig. 2 *a*, Identification of MMTV proviruses in clones of infected rat cells. Rat (XC) cells⁴⁶ were infected with MMTV-C3H, seeded in multiple-well plates at a concentration <1 cell per well, and propagated in Dulbecco's minimal essential medium plus 10% calf serum (without added hormones). Purified DNA prepared from each clonal line (~10 µg) was digested with *Eco*RI, electrophoresed in 0.8% agarose, transferred to nitrocellulose sheets¹⁴ and hybridized with ³²P-labelled cDNA randomly transcribed from MMTV-C3H RNA^{6,23}. The figure shows an autoradiogram of an analysis of DNAs from four clones, including 8, 15 and 34, described in the text. *b*, Strategies for cloning the virus-cell junctions of the proviruses from lines 8 and 34. DNA (mg aliquots) from each line were cleaved with *Eco*RI and passed through 0.8% agarose in a fractionating gel electrophoresis apparatus⁴⁷. The eluted fractions were analysed for fragments of interest as described for *a*. To isolate the junctions from line 8, DNA from the fractions containing the 12-kilobase *Eco*RI fragment was ligated with purified Charon 4A arms and then packaged *in vitro* as described by Blattner *et al.*⁴⁸. The assembled phages were plated onto the strain DP50SupF (ref. 18) and screened for proviral inserts by the procedure of Benton and Davis⁴⁹ using MMTV ³²P-labelled cDNA_{rep}. A positively hybridizing plaque was isolated for further analysis. The insert from this phage was transferred into pBR322 using vector DNA that had been cleaved with *Eco*RI and treated with calf intestinal alkaline phosphatase. DNA from one such clone was isolated and used as a substrate for restriction cleavage analysis. For sequencing, the indicated *Sac*I fragments were purified by preparative electrophoresis through 0.8% Sea-Plaque (Marine Colloids) agarose gels, then further restricted for the construction of fine structure maps. The two junctions from the provirus in line 34 were isolated separately. DNA from fractions containing the right end 4.4-kilobase *Eco*RI fragment was ligated with purified arms from the vector λgtWES·λB, then packaged, plated and assayed as described above. After isolation, the purified insert was then transferred into pBR322 and used in restriction enzyme analysis. DNA from fractions containing the six-kilobase *Eco*RI fragment from the left end of the provirus was further digested with *Sac*I, then ligated to purified arms from the vector Charon 10 (ref. 20). Packaging, plating and screening were as described above. After preliminary characterization and preparative electrophoresis through 0.8% Sea-Plaque agarose, the inserted *Sac*I fragment was cleaved with the restriction enzyme *Sau*3A, and the cleavage products ligated to *Bam*HI-cleaved pBR322 that had been treated with calf intestinal alkaline phosphatase. After transformation of the bacterial strain HB101 (ref. 50), ampicillin-resistant recombinants were screened with a probe specific for the MMTV LTR, using the procedure of Hanahan and Meselson⁵¹, for those containing the junction.

Using this criterion, of the 45 clones analysed, 12 had two or more proviruses, 14 had a single provirus and 19 had escaped infection. Figure 2*a* shows the results of such an analysis of four of the clones carrying single proviruses. We chose three of these, designated 8, 15 and 34, for further analysis. One (clone 8) carried a truncated provirus (see Fig. 2*b*) expressed under the control of glucocorticoid hormones (our unpublished data and those of D. Robertson); the second (clone 34) contained a full-sized provirus from which little, if any, stable RNA was transcribed in the presence or absence of hormone (Fig. 2*b*, unpublished data); the third, not fully analysed here, contained a full-sized provirus under steroid control (unpublished data).

Cloning unintegrated and proviral MMTV DNA

Our choice of strategies for cloning MMTV proviral DNA was influenced by a difficulty we encountered in attempting to isolate unintegrated forms of MMTV DNAs by molecular cloning in *Escherichia coli* (similar difficulties have been encountered in

attempts to clone integrated MMTV DNA (ref. 15 and D. Ucker, personal communication)). Circular MMTV molecules were isolated from dexamethasone-stimulated rat XC cells that were chronically infected with the C3H strain of MMTV. (Heterologous cells chronically infected by MMTV generally contain 1–10 copies of unintegrated DNA, apparently synthesized by reverse transcriptase, when grown in the presence of glucocorticoids^{6,16,17}.) After partial purification by Hirt fractionation and CsCl–propidium diiodide equilibrium centrifugation^{6,16}, the viral DNA was cleaved with *Eco*RI and cloned into the vector λgtWES·λB (ref. 18). Although the virus-specific material that we ligated to the vector arms seemed to be primarily intact *Eco*RI-cleaved circular molecules, each of the positively hybridizing λgtWES·MMTV recombinants had suffered deletions or rearrangements of MMTV sequences. The deletion end points were variable, but each lesion covered a common set of MMTV sequences that lie within a region of the genome (which is designated 'poison' in Fig. 1) which probably codes for gag proteins.

To investigate this phenomenon further, we cleaved the same DNA preparation with *Pst*I, an enzyme with several recognition

sites within the MMTV genome, and cloned the fragments in the *Pst*I site of the plasmid vector pBR322. We isolated recombinants containing all the expected MMTV *Pst*I fragments except the 1-kilobase fragment containing the 'poison' sequence. We concluded from these experiments that there is a sequence within the 1-kilobase *Pst*I fragment which propagates poorly, if at all, in the strains of *E. coli* used. We cannot explain this result and have not yet isolated those sequences. In analysing integration sites, we were forced to use strategies which allowed us to circumvent this cloning difficulty.

Figure 2b shows partial restriction maps of the two proviruses and describes the cloning strategies. The provirus from clone 34 seems normal, that is, the restriction fragments generated from internal regions of the provirus were identical to those of unintegrated linear DNA. The provirus in clone 8 is abnormal in that cleavage with *Eco*RI released only a single 12-kilobase fragment which hybridized with MMTV cDNA_{rep}. Further analysis with *Pst*I, *Bam*HI, *Hind*III and *Sac*I showed that the

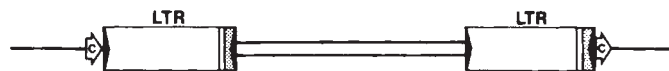


Fig. 4 Schematic diagram of an MMTV provirus. The inverted repeats at the ends of the LTRs are shown as inverted solid triangles. The direct repeat of the cell sequence at the junction site is indicated by C within the open arrow. Viral DNA is shown as a double line, flanking cellular DNA as a single line. Other symbols are as described for Fig. 1.

clone 8 provirus had incurred a large deletion of viral sequences, probably covering the *gag-pol* region of the MMTV genome; this deletion was fortuitous in that it eliminated both the poison sequence and the internal *Eco*RI site, and we were able to clone the entire steroid-responsive provirus with its flanking cell sequences in the *Eco*RI site of the λ phage vector Charon 4A (ref. 19).

The strategy for cloning the ends of the proviruses in clones 34 and 15 was dictated by the need to circumvent the poison sequence. The *Eco*RI fragments containing the right ends were cloned in λ gtWES $\cdot\lambda$ B. The *Sac*I site in the U₃ region of the LTR at the left end separates the virus-cell junction from the poison sequence; therefore the left-hand virus-cell junction from line 34 was cloned as a *Sac*I fragment in the lambda phage vector Charon 10 (ref. 20) (Fig. 2b).

Determining the boundaries of the LTR

To determine the fate of viral sequences during integration we had to know accurately the structure of viral DNA before integration. It was particularly necessary to define the boundaries of the long terminal repeat, the right and left ends of which are also the right and left ends of the linear form of unintegrated DNA (Fig. 1).

The right end should be the site of initiation of viral reverse transcription²¹, a sequence determined by direct DNA sequencing of the initial or 'strong stop' (-) strand DNA made *in vitro* (unpublished). Figure 3a shows the sequence of the region in the provirus from clone 8 covering the boundary between the left LTR and viral sequences outside the LTR, that is, the boundary between U₅ (the strong stop sequence) and the tRNA primer binding site. The strong stop transcript begins GCTGC, which is immediately preceded by 18 bases identical to the 3' end of tRNA_{Lys}²², the primer for MMTV negative strand synthesis²².

Present replication models argue that the left end of the LTR should correspond to the initiation point for (+) strand strong stop synthesis⁴. We have no direct evidence with which to define this point; therefore we have resorted to indirect arguments. There is a *Pst*I site within the LTR, near the left end of this previously described repeat unit of ~1.4 kilobases (see Fig. 1 and refs 6, 10, 23). Figure 3b shows the sequence extending leftwards from that *Pst*I site, towards the viral *Bgl*II site, which is on the 5' side of the rightward LTR. This sequence should contain the left boundary of the LTR, and within it are two features which seem useful for defining the boundary: (1) in the (+) strand there is a polypurine tract of 20 bases ending ~10 bases from the *Pst*I site; (2) a sequence of 11 base pairs, including the last nine of the polypurine tract, has precise homology with a sequence from the analogous region of avian sarcoma virus (ASV) DNA^{24,25}. Swanson *et al.*²⁴ have shown, by sequencing the junction between the LTRs of a cloned ASV circular molecule containing two copies of the LTR, that the left end of the ASV LTR is probably at the position marked with an arrow in Fig. 3b. We suggest that the left end of the MMTV LTR is at the homologous site and that the polypurine tract is involved with plus strand strong stop priming. Comparison of the proposed sequences at the left and right ends of the LTR unit show them to be related by an inverted repeat of six bases (AATGCCGC...GCGGCAGC), starting two base pairs from the ends. This supports our choice of boundaries because short inverted repeats of various lengths have been found at the

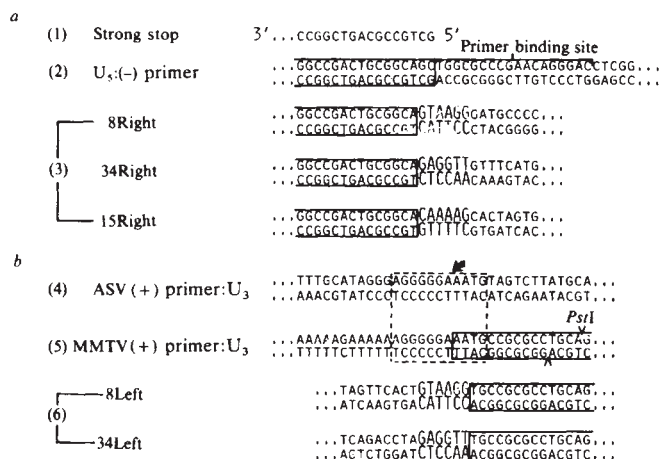


Fig. 3 a, Junctions at the right ends. The end of U₅ was determined from the sequence of 'strong stop' (-) strand DNA (RU₅) synthesized *in vitro* (1) and from the sequence of the junction between the left-hand LTR and the region encoding the binding site for tRNA_{Lys}²² (2). The latter was derived from a *Pst*I subclone of the relevant region from line 8 provirus. These sequences were then compared with the sequences of the junctions between viral and host DNA at the right ends of proviruses from lines 8, 15 and 34 (3). The sequence of strong stop DNA was determined by two methods: a modification of the di-deoxy chain termination method²² was used to analyse products synthesized by purified avian myeloblastosis virus (AMV) DNA polymerase on a template of MMTV-C3H 70S RNA; the chemical sequencing technique of Maxam and Gilbert⁵³ was used to analyse strong stop DNA synthesized by detergent-disrupted virions of MMTV-GR (provided by Dr Nurul Sarkar). For each junction sequence, both viral cell and U₅:primer binding site, DNA was labelled at a *Hpa*II site within the U₅ sequence, 30 base pairs from its right end. b, Junctions at the left ends. The end of the U₃ sequence was estimated from the position of the indicated *Pst*I site and from a comparison of sequences from the Schmidt-Ruppin A strain of ASV DNA^{24,25} (4) and from MMTV line 8 proviral DNA at the leftward boundary of the right-hand LTR (5) (see text). The 11-base homology is boxed in and the arrow indicates the boundary of the ASV U₃ region. These sequences were then compared with those from the viral-host junctions at the left ends of the two proviruses (6). The (+) primer:U₃ junction was derived from DNA labelled at the *Bgl*II site within the line 8 proviral sequences. The cell-virus junctions were derived from DNA labelled at sites within the host flanking sequences, a *Hae*III site for line 8 and a *Sau*3A site for line 34 (see Fig. 2b). Presumed viral U₅ and U₃ sequences are enclosed within solid lines and the repeated cell sequences are indicated by large letters. Sequences were obtained by the chemical cleavage method of Maxam and Gilbert⁵³, using DNA fragments that had been labelled at 3' ends either with AMV reverse transcriptase or *E. coli* DNA polymerase I.

ends of the LTRs of murine leukaemia and sarcoma viruses²⁶⁻²⁸, spleen necrosis virus (SNV)²⁹ and ASV (ref. 24).

Sequences at the host-viral junctions

Sequencing of virus-cell junctions was facilitated by transferring the *EcoRI* fragments from Charon 4A·8, λ gtWES·34R and λ gtWES·15R into the plasmid pBR322. To analyse the cell-virus junction in Charon 10·34L, the *SacI* insert was cleaved with *Sau3A* and the fragment containing the junction cloned into the *Bam*HI site of pBR322. Restriction maps derived from certain of these plasmid clones are shown in Fig. 2. Sequences from the right-end junctions were obtained by labelling at a *HpaII* site in the U₅ region and sequencing across the junction into host DNA. Figure 3 compares those sequences with that from the boundary between the right end of the leftward LTR and the tRNA primer binding site. Two proviruses are missing at least one base of U₅ sequence and the right end of the third provirus (from line 15) is missing at least two bases from the U₅ sequence. (All three probably lack the first two bases of U₅ at this end.) Also shown are the viral-host junction sequence derived from the 34L *Sau3A* subclone and that of the left junction of the provirus from line 8 derived from the *EcoRI* fragment cloned into pBR322. A comparison with the expected left end of the LTR shows that each provirus is lacking two base pairs from the proposed terminus of the LTR.

Comparison of the cellular sequences at the right and left junctions of the provirus from both lines 8 and 34 shows that there are direct repeats in the cellular sequence at the site of integration—these comprise six base pairs for both proviruses. In each case, the assignment of a GC base pair to cellular or viral sequences is ambiguous. However we argue that symmetry and consistency make a six-base pair repeat of cell DNA, with two base pairs missing from both ends of viral DNA, the logical provisional assignment.

The two base pairs removed are those which are mismatched in the inverted repeats at the ends of the LTR, and their removal leaves a perfect inverted repeat of 6 base pairs in the viral sequences at the ends of the provirus. Thus an MMTV provirus has the structure shown in Fig. 4: unique coding sequences, flanked by large terminal direct repeats, which in turn are flanked by short inverted repeats, with a direct repeat in the cell sequence at the integration site. These features are strikingly analogous to those of several transposable elements, particularly Tn9 of *E. coli*^{30,31}, *copia* of *Drosophila*³²⁻³⁴ and Ty1 of yeast³⁵⁻³⁷.

The repeated cell sequence flanking the provirus is duplicated due to integration

It has been shown for several transposable elements that the direct repeat in the host sequence which flanks the element is the result of a duplication generated during integrative recombination³⁸. To verify that a similar process is involved in retrovirus integration and to exclude the formal possibility of integration into a duplicated region of host DNA, we cloned the *EcoRI* fragment into which the provirus from line 8 was integrated. A restriction map of that fragment is indistinguishable from that of the flanking sequences in the fragment containing the line 8 provirus (Fig. 5). The sequence of the integration site shows that the six-base pair repeat which flanks the provirus in clone 8 was present only once in the integration site before insertion; thus we conclude that the duplication was generated during the integration process.

Specificity of integration sites

Analyses of proviruses of several retroviruses indicate that there must be many integration sites available for each virus in each host cell^{9,10,23,39} (see Fig. 2). However, mapping procedures are inadequate to determine whether a repeated host sequence is common to the integration sites used by any of these viruses. Figure 6 shows a comparison of the sequences for the integration

sites for MMTV proviruses in clones 8 and 34. There seem to be no common features between these sites or between either site and the ends of the proviruses. In addition, there is no obvious relationship between these sequences and those flanking the right end of the provirus in clone 15 (Fig. 3a) or those flanking the right ends of three proviruses present in a mouse mammary tumour cell¹⁵. This limited survey is thus consistent with the idea that integration occurs randomly within the host genome; however, our small sample size makes the detection of any subtle specificities unlikely. For instance, fine structure analysis of the target sites for a large number of insertions of the transposable elements Tn3 and Tn9 shows a correlation between the probability of integration and both base composition and homology with end sequences³⁸.

Models for integration of retroviral DNA

This analysis of MMTV DNA and similar analyses of other retroviral DNAs²⁷⁻²⁹ argue that any model for retroviral integration should account for the following observations: (1) A precisely defined region of viral DNA, corresponding within a few base pairs to the ends of linear DNA, seems to be invariably linked to host DNA. This implies either that a mechanism exists to recognize a specific site in viral circular DNA or that linear DNA integrates directly. (2) A limited number of base pairs, probably two, are lost from both the right and left ends of viral DNA during integration. Note that these bases are 'lost' only with respect to the probable structure of the unintegrated linear DNA. The proximal precursor to the provirus could be a circular form with a different complement of sequences in the relevant region. Furthermore, the lost base pairs do not affect the genetic

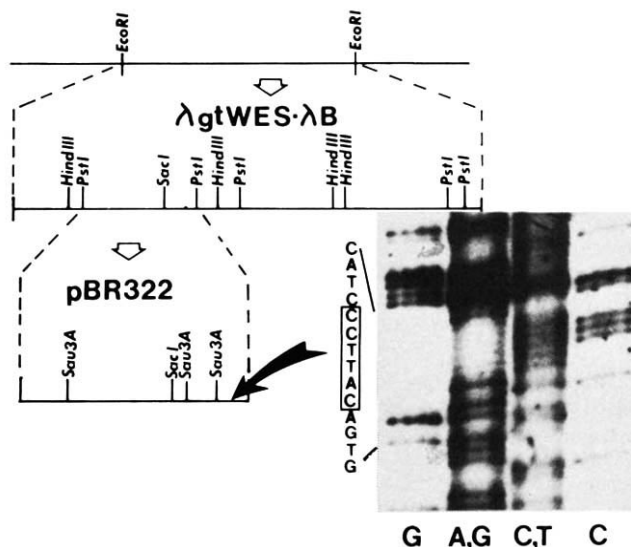


Fig. 5 The target site for the provirus in line 8. A hybridization probe made against the entire 12-kilobase *EcoRI* fragment from the line 8 provirus cloned in pBR322 was used to isolate fractions containing the target site fragment from a preparation of fractionated *EcoRI*-cleaved DNA from line 34. DNA from those fractions was ligated to purified λ gtWes· λ B arms and a recombinant phage carrying the fragment which contained the target site was isolated as described in Fig. 2 legend. The purified recombinant DNA was used for restriction enzyme cleavage analysis. For sequence analysis the *EcoRI* insert was first transferred into pBR322; the *PstI* fragment containing the target site was then sub-cloned into the *PstI* site of pBR322. The sequence was obtained by Maxam-Gilbert chemical cleavage analysis of the *Sau3A*-*PstI* fragment which had been labelled at the 3' terminus of the *Sau3A* site. The relevant part of a 10% acrylamide sequencing gel and the inferred sequence are shown. The band present in all four lanes is the result of a partial *Sau3A* cleavage 5' to the sequence CATC.


```

8      ...GTAGCAAGGGGCTAGTTCACGTAAAGGGATGCCCCAGACCA...
      ...CATCGTTCGCCGGATCAAGTGACATTCCTACGGGGTCTGGT...

34     ...AAGTGCCCACTTTAGACCTAGAGGTTGTTTCATGATTXCA...
      ...TTCACGGGCTGAAAGTCTGGATCTCCACAAAGTACTAAXGT...

```

Fig. 6 A comparison of two targets for MMTV integration into rat XC cell DNA. The sequence of the integration site used for the provirus in line 8 was determined directly as described in Fig. 5 legend. The sequence of the site used in line 34 was deduced from the sequences flanking the ends of the provirus (Fig. 3).

capacity of the provirus because they originate from regions which are repeated in linear DNA but present uniquely in viral RNA (see Fig. 1). (3) Host sequences are duplicated at the site of integration. (4) Viral DNA terminates with short inverted repeats. (5) A large number of regions in host DNA, including sites with sequences unrelated to each other or to viral DNA, can accommodate proviral DNA.

Although proviral elements from all retroviruses analysed have shown these general features, there are potentially significant differences in their fine structures: (1) The terminal base pairs which are lost from MMTV DNA after integration are not included within terminal inverted repeat sequences, whereas those from other retroviral DNAs are part of an inverted repeat²⁷⁻²⁹. (2) The size of the inverted repeats in proviral DNA varies from 20 base pairs (with four mismatches) in Moloney murine leukaemia virus (Mo-MuLV) DNA²⁸ to three base pairs in SNV DNA²⁹. (3) The length of duplication in cellular sequences generated by integration seems to be specific for each retrovirus; thus, Moloney strains of MuLV and murine sarcoma virus (MSV) generate a four-base pair repeat^{27,28}, SNV generates a five-base pair repeat²⁹ and MMTV probably generates a six-base pair repeat.

The duplication of cellular sequences strongly suggests that an early step in the integrative process will prove to be the introduction of a staggered scission in host chromosomal DNA, as proposed for transposable elements^{40,41}. The lack of homology between the two MMTV integration sites described here indicates that the enzyme responsible for this nucleolytic activity may have little sequence specificity. No such host or virus-coded activity has yet been described, although an endonucleolytic activity, encoded in the viral *pol* gene, has been found in virions of avian myeloblastosis virus⁴².

Unfortunately, our structural studies shed little light on the identity of the viral integrative precursor. Both the linear and the two circular forms of unintegrated DNA can be accommodated as precursors within credible models for integration²⁸, each of which is closely related to that proposed by Shapiro⁴¹ for the transposition of bacterial transposons. Recent work on the mechanism of restriction of MuLV replication by alleles at the *Fv-1* locus can be interpreted as supporting a circular pre-

cursor^{43,44}. In some cases, *Fv-1* alleles inhibit both the accumulation of circular forms of MuLV DNA and the integration of MuLV DNA into mouse DNA. However, it is possible that these two observations both reflect the defective nature of linear DNA; thus the identity of the proximal precursor to the provirus remains an open issue.

Functional implications of proviral structure

The significance of the structural similarities between proviruses and transposable elements (Fig. 4) is uncertain in the absence of mechanistic details about retrovirus integration or about transposition of bacterial or eukaryotic transposons. Duplications at the sites of integration argue that the generation of staggered single-stranded scissions in host DNA is an early event in both processes. Terminal inverted repeats should allow an enzyme with a single recognition specificity to operate on both ends of an integrating molecule; however, this feature may not be essential for transposition because the Ty1 elements of yeast lack terminal inverted repeats^{36,37}.

There is no information concerning the possibility that proviruses can be relocated at new sites in host genomes by means other than transcription into RNA, subsequent reverse transcription and reintegration. Experiments to test for direct transposition are difficult to design and unlikely to yield unambiguous results. However, there is evidence to suggest that retroviruses can show other forms of behaviour observed for transposable elements, including deletion formation, insertional mutagenesis and promotion of transcription into flanking cellular DNA⁴⁵. Each of these may have significant genetic consequences for the host, apart from the expression of viral genes.

The structural studies reported here do not directly further our understanding of hormonal regulation or carcinogenesis in MMTV-infected cells. However, the provirus from clone 8 is under glucocorticoid control and has been cloned intact with large regions of flanking cellular DNA; it should serve as a useful reagent for examining the role of viral and context (host) sequences in the mediation of the hormone response. The sequence of the MMTV LTR (studies to be presented elsewhere) includes an A+T-rich region characteristic of putative eukaryotic promoters. This region, which reads TATAAAAGA, is positioned 24–32 bases from the probable initiation site for RNA synthesis, the site corresponding to the 5' terminus of genomic RNA. Thus we favour the notion that LTRs, like repeated units in transposons, may provide signals for gene expression²⁴⁻²⁹.

We thank Bertha Cook for secretarial assistance. This work was supported by USPHS grants CA 12705, CA 19289, Training Grant 1T32 CA 09043 and American Cancer Society VC-70. J.E.M. was supported by a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

Received 28 August; accepted 13 November 1980.

- Bishop, J. M. *A. Rev. Biochem.* **47**, 35–88 (1978).
- Shank, P. R. *et al. Cell* **15**, 1383–1396 (1978).
- Hsu, T. W., Sahran, J. L., Mark, G. E., Guntaka, R. V. & Taylor, J. M. *J. Virol.* **28**, 810–818 (1978).
- Gilboa, E., Mitra, S. W., Goff, S. & Baltimore, D. *Cell* **18**, 93–100 (1979).
- Shank, P. R. & Varmus, H. E. *J. Virol.* **25**, 104–114 (1978).
- Shank, P. R., Cohen, J. C., Varmus, H. E., Yamamoto, K. R. & Ringold, G. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2112–2116 (1978).
- Yoshimura, F. K. & Weinberg, R. A. *Cell* **16**, 323–332 (1979).
- Kung, H. J., Shank, P. R., Bishop, J. M. & Varmus, H. E. *Virology* **103**, 425–433 (1980).
- Hughes, S. H. *et al. Cell* **15**, 1397–1410 (1978).
- Ringold, G. M., Shank, P. R., Varmus, H. E., Ring, J. & Yamamoto, K. R. *Proc. natn. Acad. Sci. U.S.A.* **76**, 665–669 (1979).
- Moore, D. H. *et al. Adv. Cancer Res.* **29**, 347–418 (1979).
- Varmus, H. E., Ringold, G. & Yamamoto, K. R. in *Glucocorticoid Hormone Action* (eds Baxter, J. D. & Rousseau, G. G.) 253–278 (Springer, Berlin, 1979).
- Fine, D. L., Plowman, J. K., Kelley, S. P., Arthur, L. O. & Hillman, E. A. *J. natn. Cancer Inst.* **52**, 1881–1886 (1974).
- Southern, E. M. *J. molec. Biol.* **98**, 503–517 (1975).
- Hager, G. L. & Donehower, L. A. in *Animal Virus Genetics: ICN-UCLA Symp. molec. cell. Biol.* (eds Fields, B., Jaenisch, R. & Fox, C. F.) (Academic, New York, in the press).
- Ringold, G. M., Yamamoto, K. R., Shank, P. R. & Varmus, H. E. *Cell* **10**, 19–26 (1976).
- Ringold, G. M., Shank, P. R. & Yamamoto, K. R. *J. Virol.* **26**, 93–101 (1978).
- Leder, P., Tiemeier, D. & Enquist, L. *Science* **196**, 175–177 (1977).
- Blattner, F. R. *et al. Science* **196**, 161–169 (1977).

- deWet, J. R. *et al. J. Virol.* **33**, 401–410 (1980).
- Taylor, J. M. *Biochim. biophys. Acta* **473**, 57–72 (1977).
- Peters, G. & Glover, C. C. *J. Virol.* **35**, 31–40 (1980).
- Cohen, J. C., Shank, P. R., Morris, V. L., Cardiff, R. & Varmus, H. E. *Cell* **16**, 333–346 (1979).
- Swanstrom, R., DeLorbe, W., Bishop, J. M. & Varmus, H. E. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Czernilofsky, A. P., DeLorbe, W., Swanstrom, R., Varmus, H. E. & Bishop, J. M. *Nucleic Acids Res.* **8**, 2967–2984 (1980).
- Sutcliffe, J. G., Shinnick, T. M., Verma, I. M. & Lerner, R. A. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3302–3306 (1980).
- Dhar, R., McClements, W. L., Enquist, L. W. & Vande Woude, G. F. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3937–3941 (1980).
- Shoemaker, C. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 3932–3936 (1980).
- Shimotomono, K., Mizutani, S. & Temin, H. M. *Nature* **285**, 550–554 (1980).
- MacHattie, I. A. & Jackowski, J. B. in *DNA Insertion Elements, Plasmids and Episomes* (eds Bukhari, A. I., Shapiro, J. A. & Adhya, S. L.) 219–228 (Cold Spring Harbor Laboratory, New York, 1977).
- Johnsrud, L., Calos, M. & Miller, J. H. *Cell* **15**, 1209–1219 (1978).
- Strobel, E., Dunsmuir, P. & Rubin, G. M. *Cell* **17**, 429–439 (1979).
- Dunsmuir, P., Brorin, W. J. Jr, Simon, M. A. & Rubin, G. M. *Cell* **21**, 575–579 (1980).
- Levis, R., Dunsmuir, P. & Rubin, G. M. *Cell* **21**, 581–588 (1980).
- Cameron, J. R., Loh, E. Y. & Davis, R. W. *Cell* **16**, 739–751 (1979).
- Farabaugh, P. J. & Fink, G. R. *Nature* **286**, 352–356 (1980).
- Gafner, J. & Phillipsen, P. *Nature* **286**, 414–418 (1980).
- Calos, M. P. & Miller, J. H. *Cell* **20**, 579–595 (1980).
- Steffen, D. & Weinberg, R. A. *Cell* **15**, 1003–1010 (1978).

40. Grindley, N. D. F. & Sherratt, D. J. *Cold Spring Harb. Symp. quant. Biol.* **43**, 1257–1268 (1978).
41. Shapiro, J. A. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1933–1937 (1979).
42. Golomb, M. & Grandgenett, D. P. *J. biol. Chem.* **254**, 1606–1613 (1979).
43. Yang, W. K. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 2994–2998 (1980).
44. Jolicouer, P. & Rassart, E. *J. Virol.* **33**, 183–195 (1980).
45. Majors, J. E. *et al. Cold Spring Harb. Symp. quant. Biol.* **45** (in the press).
46. Svoboda, J. *Nature* **186**, 980–981 (1960).
47. Leder, P. *et al. Cold Spring Harb. Symp. quant. Biol.* **42**, 915–920 (1978).
48. Blattner, F. R. *et al. Science* **202**, 1279–1288 (1978).
49. Benton, W. D. & Davis, R. W. *Science* **196**, 180–182 (1977).
50. Boyer, H. W. & Roulland-Dussoix, D. *J. molec. Biol.* **41**, 459–472 (1969).
51. Hanahan, D. & Meselson, M. *Gene* **10**, 63–68 (1980).
52. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5463–5467 (1977).
53. Maxam, A. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **74**, 560–564 (1977).

Nucleotide sequence and formation of the transforming gene of a mouse sarcoma virus

Charles Van Beveren, Janice A. Galleshaw, Vivian Jonas, Anton J. M. Berns*, Russell F. Doolittle†, Daniel J. Donoghue & Inder M. Verma

Tumor Virology Laboratory, The Salk Institute, PO Box 85800, San Diego, California 92138

†Department of Chemistry, University of California, San Diego, La Jolla, California 92093

The complete nucleotide sequence of the transforming gene of a mouse sarcoma virus has been determined. It codes for a protein of 374 amino acids. The nucleotide sequence of the junctions between a murine leukaemia virus and cellular sequences leading to the formation of the viral transforming gene have also been elucidated. The viral transforming sequence and its cellular homologue share an uninterrupted stretch of 1,159 nucleotides, with few base substitutions. The predicted amino acid sequence of the mouse sarcoma virus transforming gene was found to share considerable homology with the proposed amino acid sequence of the avian sarcoma virus oncogene (src) product.

MANY of the retroviruses capable of cellular transformation have acquired a normal cellular sequence as an integral part of their genome¹. The acquired cellular sequences can be expressed either as an independent viral gene or as a product fused with viral structural proteins^{2–10}. With the exception of Rous sarcoma viruses (RSV), acquisition of cellular sequences occurs at the expense of viral genomic sequences required for replication^{1,11–18}. Not surprisingly, most retroviruses capable of inducing cellular transformation are unable to propagate without helper viruses.

We were interested in the biogenesis of a transforming gene at the level of nucleotide sequences. We chose to study Moloney mouse sarcoma virus (Mo-MSV) which is able to transform fibroblasts *in vitro* and induce neoplasia *in vivo*, but is unable to replicate^{19–21}. It was obtained from sarcomas induced in BALB/c mice after passage of high doses of Moloney murine leukaemia virus (Mo-MLV)²². The genome of a cloned isolate of Mo-MSV (clone 124, ref. 23) has been characterized extensively. Briefly, the Mo-MSV genomic RNA is about 5.3 kilobases long and shares 60–70% sequence homology with Mo-MLV²⁴. Figure 1 shows a schematic heteroduplex formed between a genome-length Mo-MLV cDNA transcript and Mo-MSV 124 genomic RNA. The Mo-MSV genome has a sequence substitution of about 1.2 kilobases and two deletions of approximately 1.2 and 1.6 kilobases. About 2.0 kilobases at the 5' end and 0.6–0.7 kilobases at the 3' end are shared between the two viruses^{11–15}.

We have molecularly cloned the unintegrated circular forms of Mo-MSV clone 124 and Mo-MLV DNA in bacteriophage λ (refs 25, 26), by using molecularly cloned Mo-MSV^{src}-specific DNA fragments as probe, a 14.0-kilobase *EcoRI* fragment from uninfected mouse cells was identified and subsequently molecularly cloned in bacteriophage λ (refs 27, 28). The cloned cellular DNA fragment contains an uninterrupted stretch of about 1.1 kilobases homologous to Mo-MSV-specific sequences. The Mo-MSV^{src}-specific sequences will be referred to here as *v-mos*^{Mo} [viral (v), mouse sarcoma virus (*mos*)],

Moloney (Mo)] and their cellular homologue is referred to as *c-mos*^{Mo} [cellular (c)]. We were interested in determining the nucleotide sequence in the region of the junctions of *c-mos*^{Mo} and Mo-MLV sequences to learn more about the process leading to the formation of the *v-mos*^{Mo} gene. We present here the following results: (1) the complete nucleotide sequence of the *v-mos*^{Mo} gene of Mo-MSV clone 124; (2) the predicted amino acid sequence of the *v-mos*^{Mo} gene product; (3) nucleotide sequences of the 5' and 3' extremities of *c-mos*^{Mo} and Mo-MLV leading to the formation of the *v-mos*^{Mo} gene; (4) nucleotide sequence comparison of the portions of *v-mos*^{Mo} and *c-mos*^{Mo}; (5) comparison of the predicted amino acid sequence of the *v-mos*^{Mo} gene product with the proposed avian sarcoma virus oncogene (*src*) product.

Construction of plasmids

The following plasmids were constructed for the purpose of determining the nucleotide sequence of the appropriate regions (Fig. 2). The plasmid, p·MSV-1, contains the entire Mo-MSV DNA, whereas the plasmid, p·MSV-21, contains a majority of the *v-mos*^{Mo} gene sequences. The cellular DNA fragment containing the *c-mos*^{Mo} sequence was cloned in bacteriophage Charon 4A and portions of it were subsequently subcloned in the plasmid pBR322. The plasmid, p·CS-1, contains the 5' half of the *c-mos*^{Mo} and cellular sequences, whereas p·CS-2 contains the entire coding region of *c-mos*^{Mo} and adjacent cellular sequences. The plasmid, p·MLV-1, contains the entire Mo-MLV genome. The approximate position of the junction of Mo-MLV and *v-mos*^{Mo} sequences was obtained from the heteroduplex map (Fig. 1) and physical maps generated by restriction endonucleases^{29,30}.

Nucleotide sequences of the *v-mos*^{Mo} gene

The Maxam–Gilbert method of DNA sequencing was used. In general, 5'→3' sequences were determined by labelling with polynucleotide kinase and [γ -³²P]ATP, and the 3'→5' sequences were determined by labelling the 3' ϵ with [α -³²P]CTP and terminal transferase. The complete nucleotide

* Present address: Laboratory of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands.

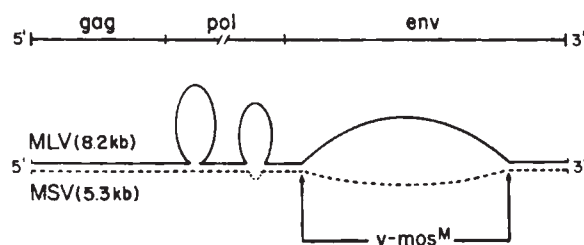


Fig. 1 Schematic heteroduplex formed between genome-length Mo-MLV DNA and Mo-MSV 124 genomic RNA¹¹. kb, Kilobases.

sequence of a DNA fragment encompassing the *v-mos*^{Mo} gene of Mo-MSV 124 is shown in Fig. 3. The nucleotide sequence from just before the first *Bgl*I site through the *Hind*III site (1,356 base pairs) was obtained from p·MSV-21 (Fig. 2). The nucleotide sequence of *v-mos*^{Mo} past the *Hind*III site was determined from p·MSV-1.

There is only one major region in which a reading frame in either direction is open for translation. It starts with an ATG about 10 nucleotides downstream from the single *Xba*I site (Fig. 2), and can encode a protein containing 374 amino acids. The open reading frame terminates with an opal codon (TGA) within the acquired cellular sequences. Figure 3 also shows the predic-

ted amino acid sequence of the *v-mos*^{Mo} gene product. Based on the predicted sequence, the largest possible *v-mos*^{Mo} gene product has a molecular weight of 40,992, which is in good agreement with the estimated size of a 37,000 molecular weight (37 K) protein and a related family of proteins synthesized in an *in vitro* translation of Mo-MSV clone 124 genomic RNA³¹. The synthesis of the 37K protein and a related family of polypeptides is specifically inhibited when the Mo-MSV 124 genomic RNA is preannealed to *v-mos*^{Mo}-specific DNA fragments (J. Papkoff, T. Hunter and I. M. V., unpublished results). The synthesis of other viral proteins, like the p60 *gag* gene product, is not inhibited by the *v-mos*^{Mo}-specific fragments. Note, however, that the 37K and related polypeptides synthesized *in vitro* have not been conclusively shown to be *in vivo* *v-mos*^{Mo} gene products, as no antisera against these polypeptides are yet available. Based on its amino acid sequence, the *v-mos*^{Mo} gene product has a net positive charge, in agreement with the mobility of the *in vitro* 37K protein on isoelectric focusing gels (J. Papkoff and T. Hunter, personal communication).

Nucleotide sequence of the *c-mos*^{Mo} and Mo-MLV junctions

To determine the nucleotide sequences at the 5' and 3' junctions of the *c-mos*^{Mo} and Mo-MLV leading to the formation of the *v-mos*^{Mo} gene, we used DNA fragments from the plasmids p·CS-1, p·CS-2 and p·MLV-1 (Fig. 2). Figure 4 shows the

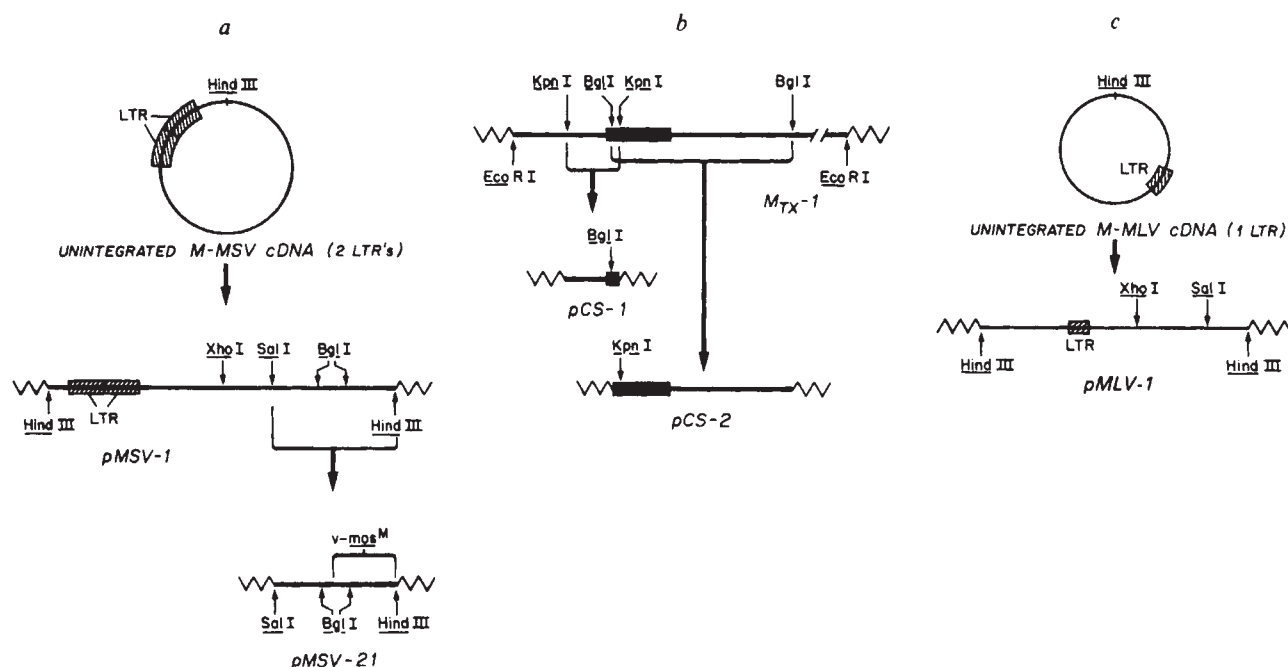


Fig. 2 Construction of plasmids. *a*, p·MSV-1 and p·MSV-21. The unintegrated circular forms of Mo-MSV DNA from freshly infected NIH/3T3 cells were isolated and cleaved at the unique *Hind*III site³⁰. The DNA was molecularly cloned in the unique *Hind*III site of bacteriophage Charon 21A as described elsewhere²⁵. The insert DNA containing the entire Mo-MSV DNA with two long terminal repeats was subcloned in the *Hind*III site of plasmid pBR322. About 1.0 µg of p·MSV-1 was cleaved with restriction endonucleases *Hind*III and *Sal*I and fractionated on 0.7% agarose gels. The 2.2-kilobase *Hind*III-*Sal*I fragment containing the *v-mos*^{Mo} gene was eluted from the gel and joined to *Hind*III- and *Sal*I- cleaved pBR322 by T4 DNA ligase as described elsewhere²⁵. The recombinant plasmids were used to transform *Escherichia coli* C600SF and colonies were screened both for drug resistance (*Amp*^R *Tet*^S) and hybridization to Mo-MSV cDNA probes. The bracket denotes the *v-mos*^{Mo} sequences. *b*, p·CS-1 and p·CS-2. The 14.0-kilobase *Eco*RI fragment from uninfected NIH/3T3 cells containing the *c-mos*^{Mo} sequences was cloned in bacteriophage Charon 4A. The recombinant clone, λ·*M_{TX}-1*, was analysed by restriction endonuclease²⁸. The clone, λ·*M_{TX}-1*, was cleaved with *Kpn*I which generated an expected fragment of about 1.2 kilobases, containing the 5' half of *c-mos*^{Mo} and adjacent cellular sequences. It was eluted from an agarose gel and a stretch of oligo(dC) residues was added using terminal transferase, as described previously⁴³, and cloned in the *Pst*I-cleaved [dG]-tailed pBR322. To make the plasmid p·CS-2, the recombinant clone, λ·*M_{TX}-1*, was cleaved with *Bgl*I and a 5.5-kilobase fragment containing the 3' *c-mos*^{Mo} and adjacent cellular sequences was isolated from agarose gels and subcloned in the plasmid pBR322 by the [dC]-[dG]-tailing method⁴³. The solid box indicates the *c-mos*^{Mo} sequences. *c*, p·MLV-1. The details of molecular cloning of unintegrated circular forms of Mo-MLV are described elsewhere²⁶. Briefly, the Mo-MLV DNA was cleaved at a unique site with *Hind*III and cloned in the unique *Hind*III site of bacteriophage Charon 21A. The recombinant clone containing the Mo-MLV DNA was cleaved with *Hind*III and the insert Mo-MLV DNA further subcloned in the *Hind*III site of pBR322. The orientation of the insert in the plasmid was determined by restriction endonuclease analysis. The wavy lines show vector DNA.

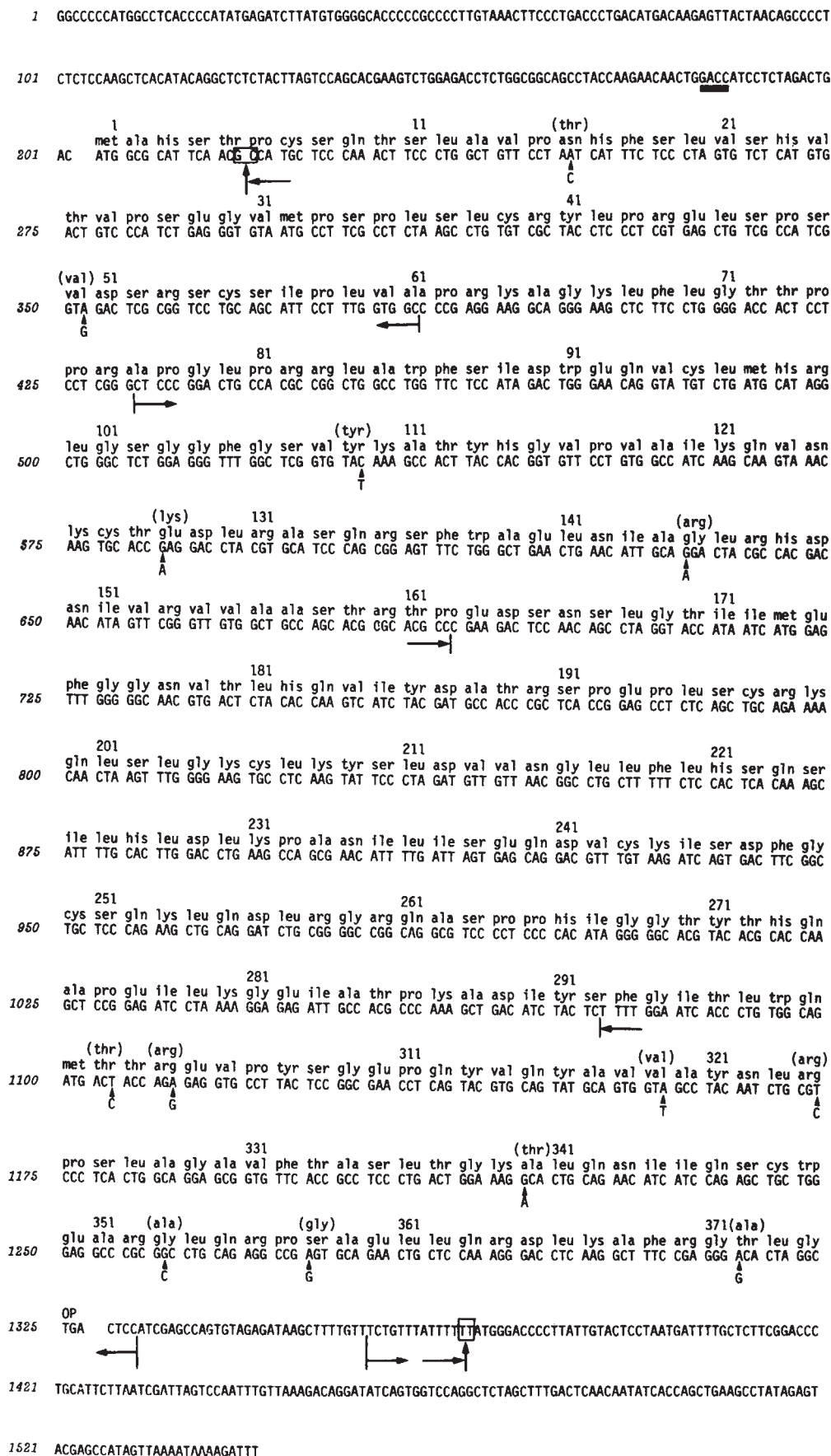
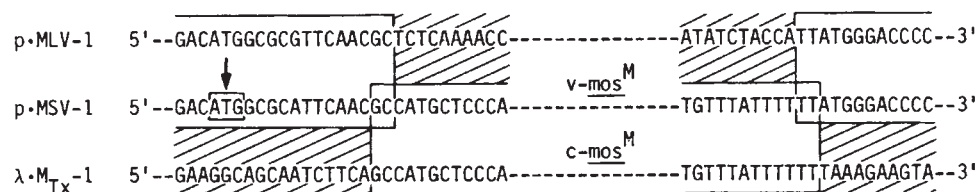


Fig. 3 Nucleotide and predicted amino acid sequence of *v-mos*^{Mo} gene. The complete nucleotide sequence of Mo-MSV DNA fragment containing the *v-mos*^{Mo} gene is shown. The numbers in italics on the left-hand margin refer to the nucleotide numbers (1–1,547). The numbers in the figure (1–374) refer to the predicted amino acid sequence of the *v-mos*^{Mo} putative gene product, starting with Met as number 1 and terminating with an opal codon, TGA. The thick solid arrows under the boxed nucleotides show the recombination junctions between *c-mos*^{Mo} and Mo-MLV sequences; the horizontal arrows indicate the regions of the *c-mos*^{Mo} whose nucleotide sequence was determined; the solid triangles and the nucleotides under them denote the base alterations in the *c-mos*^{Mo} sequences; the amino acids in parentheses above the predicted amino acid sequences of *v-mos*^{Mo} gene product are the predicted changes in the *c-mos*^{Mo} product; the closed bar between nucleotides 184 and 187 shows the position of the 192-nucleotide Mo-MLV sequence deleted in Mo-MSV. The locations of some endo-R cleavage sites are: *AcyI*: 986; *AvaI*: 385, 426; *BalI*: 557; *BglI*: 8,448; *BglII*: 26; *Clal*: 1,432; *HhaI*: 208, 681; *HindIII*: 1,351; *Hinfl*: 353, 692, 739, 1,083, 1,326, 1,486; *HpaI*: 844; *HpaII*: 435, 448, 776, 979, 1,028, 1,125; *KpnI*: 711; *PstI*: 368, 794, 966, 1,227, 1,266; *PvuII*: 789, 1,504; *SsrII*: 1,258; *XbaI*: 192. A complete list of endo-R restriction sites for this sequence is available on request.

Fig. 4 The 5' and 3' recombination junctions between Mo-MLV, Mo-MSV and λ -M_{TX}-1. The 5' and 3' boundaries of the recombination junctions between Mo-MLV and *c-mos*^{Mo} leading to the formation of the *v-mos*^{Mo} gene sequence are shown. The open boxes denote homology whereas the hatched areas indicate non-homology. The ATG (below the arrow) indicates the position of the predicted first amino acid (Met) of the *v-mos*^{Mo} gene product (Fig. 3).



sequence of *c-mos*^{Mo} and p·MLV-1 at the 5' and 3' ends of the *v-mos*^{Mo} gene sequence. The 5' junction of *c-mos*^{Mo} with Mo-MLV occurs 12 nucleotides downstream from the initiation codon of the putative *v-mos*^{Mo} gene product. At the 3' end of the junction there are 19 nucleotides to the right of the *Hind*III site at the end of a stretch of six T residues. The N-terminus of the *v-mos*^{Mo} gene product extends five amino acids into the Mo-MLV genome past the *c-mos*^{Mo} junction. There is no direct sequence homology between Mo-MLV and *c-mos*^{Mo} junction sequences. However, the sequence TTCA is present immediately to the left of the 5' junction in *c-mos*^{Mo}, and two nucleotides to the left of the 5' junction in Mo-MLV (Fig. 4). This kind of sequence homology may suggest that *v-mos*^{Mo} originated by homologous recombination between *c-mos*^{Mo} and Mo-MLV sequences. The exact mechanism of recombination, however, remains obscure.

Comparison of portions of *c-mos*^{Mo} and *v-mos*^{Mo} sequences

It was of interest to determine the sequence divergence between *c-mos*^{Mo} and *v-mos*^{Mo} sequences. A cloned isolate of Mo-MLV (Mo-MLV clone 1) has been obtained^{32,33}. We have propagated Mo-MSV clone 124 and Mo-MLV (clone 1) in our laboratory since 1973. Figure 3 compares the nucleotide sequence of portions of *v-mos*^{Mo} and *c-mos*^{Mo} sequences. In a stretch of about 500 nucleotides at the 5' end there seem to be only five

base changes. Similarly, in a stretch of 300 nucleotides at the 3' end there are only eight single base substitutions. The nucleotide sequence homology between the *v-mos*^{Mo} and *c-mos*^{Mo} sequences is in complete agreement with the results obtained from nuclease S₁ analysis of hybrids formed between cloned λ ·M_{TX}-1 DNA (containing *c-mos*^{Mo} sequences, Fig. 2) and Mo-MSV genomic RNA²⁸. The initiation codon ATG (at position 203 in Fig. 3) of the longest possible *v-mos*^{Mo} gene product is not present in the *c-mos*^{Mo} sequences (Fig. 4). The sequences in *c-mos*^{Mo} alone are not able to transform cells; however, when portions of Mo-MLV containing the long terminal repeat (LTR) are attached to it, cellular transformation can be observed (W. M. McClements and G. F. van de Woude, personal communication). Thus, it seems that the proteins encoded by *v-mos*^{Mo} and *c-mos*^{Mo} sequences are very similar.

The nucleotide sequences adjacent to the 3' end of the *v-mos*^{Mo} gene in Mo-MLV and Mo-MSV are identical. However, at the 5' end, 29 nucleotides from the 5' junction of *c-mos*^{Mo} and Mo-MLV, the Mo-MSV seems to have undergone a previously undetected deletion of 192 nucleotides (shown as closed bar in Fig. 3). Analysis of the sequences on the 5' and 3' ends of the deletion reveals a common sequence, GACC. This small deletion was not observed in the heteroduplexes formed between Mo-MLV cDNA and Mo-MSV 124 RNA^{11,15}. As there are only 29 base pairs between this deletion and the major substitution loop, it was perhaps scored as a part of the major substitution loop, β_5 (ref. 11).

v-mos^M

ASV src

1

MAH

S

T

P

C

S

Q

T

S

L

A

V

P

N

H

F

S

L

V

S

H

V

T

V

P

S

E

G

V

M

P

S

P

L

188

...

S

P

V

S

D

F

D

N

A

K

G

L

N

V

K

H

Y

K

I

R

K

L

D

S

G

G

F

Y

I

T

S

R

T

Q

L

Q

37

S

L

C

R

Y

L

P

R

E

L

S

P

S

V

D

S

R

S

C

S

I

P

L

V

A

P

R

K

A

G

K

L

F

L

G

T

224

K

A

C

R

Q

L

V

A

Y

Y

S

K

H

A

D

G

L

C

H

R

L

T

N

V

C

P

T

73

T

P

P

R

A

P

G

L

P

R

R

L

A

W

F

S

I

D

W

E

Q

V

C

L

M

H

R

L

G

S

G

G

F

G

S

V

251

S

K

P

Q

T

Q

G

L

A

K

D

A

W

E

I

P

R

D

V

A

A

A

G

G

E

A

G

A

G

L

L

W

R

G

L

D

109

Y

K

A

T

Y

H

G

V

P

V

A

I

K

Q

V

N

K

C

T

E

D

L

R

A

S

Q

R

S

F

W

A

E

L

N

I

A

287

G

D

L

E

R

H

H

Q

S

G

H

K

D

S

E

A

R

H

H

V

P

G

A

F

L

Q

E

A

145

G

Fig. 5 Comparison between the proposed amino acid sequences for the *v-mos*^{Mo} and ASV *src* gene products. The amino acid sequences of the *v-mos*^{Mo} (Fig. 3) and ASV *src* (ref. 36) gene products were aligned for optimum identity as described elsewhere^{37,38}. The portions of the sequences presented here include the entire *v-mos*^{Mo} sequence, and ASV *src* sequence from amino acid 188 to its carboxy terminus (residue 530). Areas of identity are depicted by boxes. The letter code for the amino acids is: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

The sequence in Fig. 3 was examined for features which have been implicated in RNA synthesis in other systems. Neither the *v-mos*^{Mo} nor the *c-mos*^{Mo} sequences seem to contain the canonical sequence (TATAAA, Hogness-Goldberg box) often implicated in promotion of transcription. Examination of the A+T-rich region of the sequence shown in Fig. 3 reveals a sequence, TTGTAAA, beginning at nucleotide 53, which might be used as a DNA-dependent RNA polymerase II binding site. Further sequence analysis of Mo-MLV, 5' from nucleotide 1, reveals another potential promoter sequence (data not shown). The importance of the TATAAA sequence is uncertain, however, as it has recently been reported that removal of TATAAA sequences does not eliminate the transcription of histone genes *in vivo*³⁴. To the 3' side of the *v-mos*^{Mo} coding region, ~210 nucleotides from the TGA stop codon, there is the sequence AATAAA which has been associated with the addition of poly(A) tails onto eukaryotic mRNAs³⁵.

Comparison with other proteins

The amino acid sequence of the *v-mos*^{Mo} gene product, as derived from the DNA sequence, was subjected to a computer search for sequence resemblance among ~1,300 known amino acid sequences, including those for the T-antigens of polyoma and SV40. Although several sequences were found to have some marginal resemblances to portions of the *v-mos*^{Mo} gene product, only the recently reported oncogene product (deduced from DNA sequence studies) of avian sarcoma virus (ASV)³⁶ had a genuinely homologous sequence.

A very significant homology was uncovered when overlapping segments of the two proposed amino acid sequences were compared (Fig. 5). Residues 1–374 of the *v-mos*^{Mo} gene product were placed into optimum alignment with residues 188–530 (C-terminus) of the ASV *src* gene product using a program which has an algorithm similar to that described by Needleman and Wunsch^{37,38}, and which uses an empirically derived gap penalty. After an alignment score had been obtained, the two sequences were randomly jumbled, 36 comparisons of jumbled sequences executed and the alignment scores again computed. A mean value of the alignment scores was, finally, determined and the standard deviation calculated. The alignment score obtained for the two authentic sequences was about 7 s.d. above the mean of the jumbled comparisons, a highly significant result³⁸.

The optimum alignment of the two sequences revealed that they are about 23% identical in the region of the 371-residue overlap shown in Fig. 5. The strongest homology occurs over the stretch of the *v-mos*^{Mo} sequence corresponding to residues 227–309 and the ASV sequence residues 387–468. In this region there are 36 identical residues over the course of the 83-residue span, or about 45% identity. The overall degree of resemblance is approximately the same as that obtained in a comparison of mouse haemoglobin α -chain and hagfish

haemoglobin³⁹, or of the β -chain of human haemoglobin⁴⁰ and human myoglobin⁴¹. The murine and avian haemoglobin α -chains, by contrast, have evolved less rapidly than have the proposed murine and avian viral proteins: the mouse haemoglobin α -chain has about 70% identity with chicken α -haemoglobin.

No detectable nucleotide sequence homology was observed when ASV *src*-specific cDNA was hybridized to viral RNA obtained from the Mo-MSV(MLV) complex⁴². This result was confirmed by a computer comparison of the nucleotide sequences of *v-mos*^{Mo} (Fig. 3) and the ASV *src* gene product³⁶. However, the amino acid sequence homology between the oncogene products of ASV and Mo-MSV indicates that there can be little doubt that the two proteins have evolved from a common ancestor.

Conclusion

We have studied the biogenesis of the transforming gene of Mo-MSV. The Mo-MSV was isolated from sarcomas obtained after passage of Mo-MLV in mice. The Mo-MLV acquired cellular sequences, and underwent several deletions to yield a transforming but replication-defective virus. We have determined the entire nucleotide sequence of the Mo-MSV-specific sequences, referred to as the *v-mos*^{Mo} gene, in the Mo-MSV. Furthermore, the recombination junctions between the Mo-MLV and the cellular sequences leading to the formation of the *v-mos*^{Mo} gene have also been determined. The *v-mos*^{Mo} and *c-mos*^{Mo} share an uninterrupted stretch of 1,159 nucleotides with few base changes. There are no apparent structural homologies between the Mo-MLV and *c-mos*^{Mo} sequences at the point of recombination. However, there seems to be a small sequence homology, TTCA (Fig. 4), at the 5' junction of Mo-MLV and *c-mos*^{Mo}, perhaps suggesting homologous recombination. Due to the absence of specific tumour antisera, no authentic *v-mos*^{Mo} gene product has been identified. The 37K *in vitro* translation product³¹ is specifically inhibited if the RNA is hybridized to a *v-mos*^{Mo} gene DNA fragment before translation (J. Papkoff, T. Hunter and I. M. V., unpublished results). The predicted amino acid sequence of the *v-mos*^{Mo} gene product can now be used to verify the nature of the 37K protein synthesized *in vitro*. Finally, the proposed amino acid sequence of the *v-mos*^{Mo} gene product has been shown to share significant homology with the ASV *src* gene product.

We thank Bart Sefton and Walter Eckhart for reading the manuscript and Carolyn Goller for preparation of it, Drs P. Czernilofsky and Hugo Martinez for help with computer programs, Dr E. P. Reddy for communicating his sequencing data on Mo-MSV^{src}-specific fragment before publication, and M. Jones and D. Murdock for help with various stages of the study. This work was supported by grants from the NCI and the American Cancer Society. D.J.D. acknowledges a Helen Hay Whitney Foundation postdoctoral fellowship.

Received 21 July; accepted 11 November 1980.

- Bishop, J. M. *A. Rev. Biochem.* **47**, 37 (1978).
- Spector, D. H., Varmus, H. E. & Bishop, J. M. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4102 (1978).
- Duesberg, P., Bister, K. & Vogt, P. K. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4320 (1977).
- Hayman, M. J., Roy-Pokora, B. & Graf, T. *Virology* **92**, 31 (1979).
- Rettenmier, C. W., Anderson, S. M., Rieman, M. W. & Hanafusa, H. *J. Virol.* **32**, 749 (1979).
- Lee, W.-H. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 2018 (1980).
- Hanafusa, T. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 3009 (1980).
- Reynolds, F. H. Jr, van de Ven, W. J. M. & Stephenson, J. R. *J. Virol.* **28**, 665 (1978).
- Witte, O. N., Rosenberg, N., Paskind, M., Shields, A. & Baltimore, D. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2488 (1978).
- van de Ven, W. J. M., Kahn, A. S., Reynolds, F. H. Jr, Mason, K. T. & Stephenson, J. R. *J. Virol.* **33**, 1034 (1980).
- Hu, S., Davidson, N. & Verma, I. *Cell* **10**, 469 (1977).
- Chien, Y.-H. *et al. J. Virol.* **31**, 752 (1979).
- Hu, S. F., Lai, M. M. C. & Vogt, P. K. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1265 (1979).
- Shields, A., Goff, S., Paskind, M., Otto, G. & Baltimore, D. *Cell* **18**, 955 (1979).
- Donoghue, D. J., Sharpe, P. A. & Weinberg, R. A. *J. Virol.* **32**, 1015 (1979).
- Sherr, C. J., Fedele, F. A., Oskarsson, M., Maizel, J. & Van de Woude, G. *J. Virol.* **34**, 200 (1980).
- Van de Woude, G. F. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 4464 (1979).
- Mellon, P., Pawson, A., Bister, K., Martin, G. S. & Duesberg, P. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5874 (1977).
- Van Zaane, D. & Bloemers, H. P. J. *Biochim. biophys. Acta* **516**, 249 (1978).

- Aaronson, S. A. & Rowe, W. P. *Virology* **42**, 9 (1970).
- Scolnick, E. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 4650 (1975).
- Moloney, J. B. *Natn. Cancer Inst. Monogr.* **22**, 139 (1966).
- Ball, J. K., McCarter, J. A. & Sunderland, S. M. *Virology* **56**, 268 (1973).
- Dina, D., Beemon, K. & Duesberg, P. *Cell* **9**, 299 (1976).
- Verma, I. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 1773 (1980).
- Berns, A. *et al. J. Virol.* (in the press).
- Oskarsson, M., McClements, W. M., Blair, D. G., Maizel, J. V. & van de Woude, G. F. *Science* **207**, 1222 (1980).
- Jones, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **77**, 2651 (1980).
- Benz, F. D. Jr & Dina, D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 3294 (1979).
- Verma, I. M. *Nucleic Acids Res.* **6**, 1863 (1979).
- Papkoff, J., Hunter, T. & Beemon, K. *Virology* **101**, 91 (1980).
- Moloney, J. B. *J. natn. Cancer Inst.* **24**, 933 (1969).
- Fan, H. & Paskind, M. *J. Virol.* **14**, 421 (1974).
- Grosschedl, H. & Birnstiel, M. L. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1432 (1980).
- Proudfoot, N. J. & Brownlee, G. G. *Nature* **263**, 211 (1976).
- Czernilofsky, A. P. *et al. Nature* **287**, 198 (1980).
- Needleman, S. B. & Wunsch, C. D. *J. molec. Biol.* **48**, 443 (1970).
- Jue, R. A., Woodbury, N. W. & Doolittle, R. F. *J. molec. Evol.* **15**, 129 (1980).
- Liljeqvist, G., Braunitzer, G. & Paleus, S. *Hoppe-Seyler's Z. physiol. Chem.* **360**, 125 (1979).
- Braunitzer, G. *et al. Hoppe-Seyler's Z. physiol. Chem.* **325**, 283 (1961).
- Herrera, A. E. R. & Lehmann, M. *Nature new Biol.* **232**, 149 (1971).
- Stehelin, D., Guntaka, R. V., Varmus, H. E. & Bishop, J. M. *J. molec. Biol.* **101**, 349 (1976).
- Van Beveren, C., Goddard, J. G., Berns, A. & Verma, I. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 3307 (1980).

A model from electron microscopy for the molecular structure of fibrinogen and fibrin

John W. Weisel, George N. Phillips Jr & Carolyn Cohen

Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, Massachusetts 02254

The overall architecture of the fibrinogen molecule and aspects of its packing to form fibrin have been derived from a study of electron microscope images of ordered arrays. The molecule, of length 450 Å, is seen in more detail than the Hall-Slayter model and is made up of seven globular domains connected by rod-like segments.

FIBRINOGEN is the soluble precursor of the fibrin clot in the plasma. On tissue injury, an enzymatic clotting cascade is initiated leading to thrombin cleavage of the two small pairs of highly charged peptides from fibrinogen^{1,2}. The molecule then becomes highly insoluble and rapidly aggregates into the fibrin clot. This noncovalently bonded polymer is cross-linked by the fibrin-stabilizing factor enzyme and is later dissolved by cleavage with plasmin. The processes of clotting and fibrinolysis both occur continually in the normal functioning of the vascular system. The complexity of this clotting process is reflected in the complex structure of the fibrinogen molecule.

Fibrinogen has a molecular weight of 340,000 and an elongated form^{3,4} but its detailed shape is unknown. Hall and Slayter⁵ obtained electron micrographs of individual molecules, revealing a protein about 475 Å long consisting of a linear array of three globular domains (Fig. 1A). Despite considerable controversy, this 'trinodular' model is now generally accepted⁵⁻¹¹.

To account for the small 223 Å axial period of fibrin, Hall and Slayter invoked a 'shrinkage' of the molecule, but their proposal is inconsistent with results of X-ray diffraction studies, which show no large-scale changes in molecular structure on the conversion of fibrinogen to fibrin^{12,13}. The axial period of fibrin can be accounted for most simply by a half-staggered arrangement of fibrinogen monomers¹³.

Chemical studies indicate that fibrinogen is a dimer: the molecule consists of three pairs of different polypeptide chains (called A α , B β and γ) linked by 29 disulphide bridges^{14,15}. The complete amino acid sequence of human fibrinogen and the complex pattern of disulphide cross-links have recently been established¹⁶⁻²². Sequence analysis has also been used to locate certain regions of α -helical coiled coil^{23,24} that had been predicted to connect the globular domains of the molecule²⁵. However, these studies do not provide direct evidence on the overall morphology of the molecule or its packing in fibrin.

The most powerful approach to a determination of the structure of fibrinogen is X-ray crystallographic analysis. An important discovery was that microcrystals can be produced on limited cleavage of bovine fibrinogen with a bacterial protease²⁶ and that with further digestion, large single crystals, ordered to about 4 Å resolution, can be grown²⁷. Crystallographic and chemical evidence show that the modified molecule in the crystal is only 5-10% smaller in mass than native fibrinogen²⁸. Moreover, the molecules forming the crystals clot and the fibrin so produced has a band pattern in the electron microscope very similar to that of native fibrin, indicating that the modified protein is largely intact (Fig. 2D, E). (Other proteolytic enzymes have also been shown to modify fibrinogen so that microcrystals and crystals can be grown²⁹.)

Our strategy for determining the low resolution crystallographic structure of fibrinogen is the refinement of a model against X-ray data³⁰. Here we derive a more detailed molecular model for fibrinogen than the trinodular Hall-Slayter structure and describe packing arrangements that account for electron

microscope images in a variety of highly ordered arrays including fibrin.

Development of the model

It has been recognized for some time that the electron microscope band pattern of fibrin cannot be accounted for by a simple Hall-Slayter model. Within the repeat of 225 Å in negative contrast there is one bright strain-excluding band about 60 Å wide and three narrow light bands, the middle one being somewhat less prominent or variable in intensity³¹⁻³⁴ (Fig. 2D). The size and number of bands in this image show quite plainly that the globular domains in the fibrinogen molecule must be considerably smaller than those of the Hall-Slayter model (Fig. 1A) and that a trinodular structure cannot give rise to the details of the staining pattern.

A key to the interpretation of the fibrin band pattern can be found in the so-called 'orthogonal sheet' structure^{26,28} (Fig. 2A). A simple, half-staggered arrangement of this highly regular array gives a band pattern closely resembling that of negatively stained fibrin²⁸. An important aspect of the orthogonal sheet form is its extremely detailed substructure. The features are such that neither the Hall-Slayter model, nor, indeed, any simple arrangement of molecules running parallel to the microcrystal axis can readily account for this image. However, analysis of a variety of microcrystals of fibrinogen, including the orthogonal

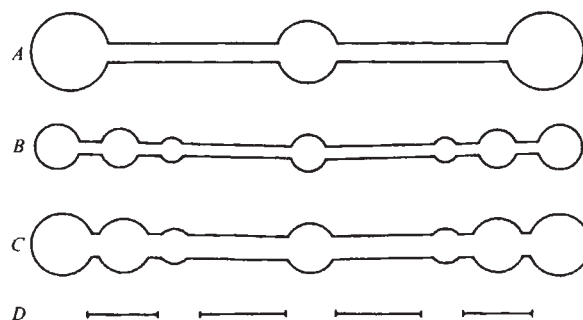


Fig. 1 Models for the fibrinogen molecule. *A*, Trinodular Hall-Slayter model, scaled to a length of 450 Å. The end domains are about 65 Å in diameter and the central region is about 50 Å. Recent micrographs show that the end domains seem to be elongated in shape^{11,43}. *B*, Fibrinogen model derived from electron micrographs of crystals and microcrystals. Diameters of the globular regions (starting from the end): 36, 32, 20, 30 Å. The diameter of the rod region has been taken as 10 Å. The molecule is not co-linear, but has a bend of a few degrees. This model from electron microscopy has about one-third the hydrated volume because of dehydration and stain penetration. *C*, Hydrated version of *B*. The dimensions have been increased so that the molecule has the volume calculated from crystallographic data (3.8×10^5 Å³)²⁹. This approximate model will be used in the initial phasing of the X-ray data. *D*, Predicted α -helical coiled-coil regions based on analysis of the amino acid sequence^{23,24}. The length of the region between two 'braces' of disulphide bonds is 111 residues, or about 160 Å; the coiled coil would begin at the central domain. We infer that the smallest globular domain may correspond to a region predicted to be non-helical where plasmin cleavage occurs²³ (see text).

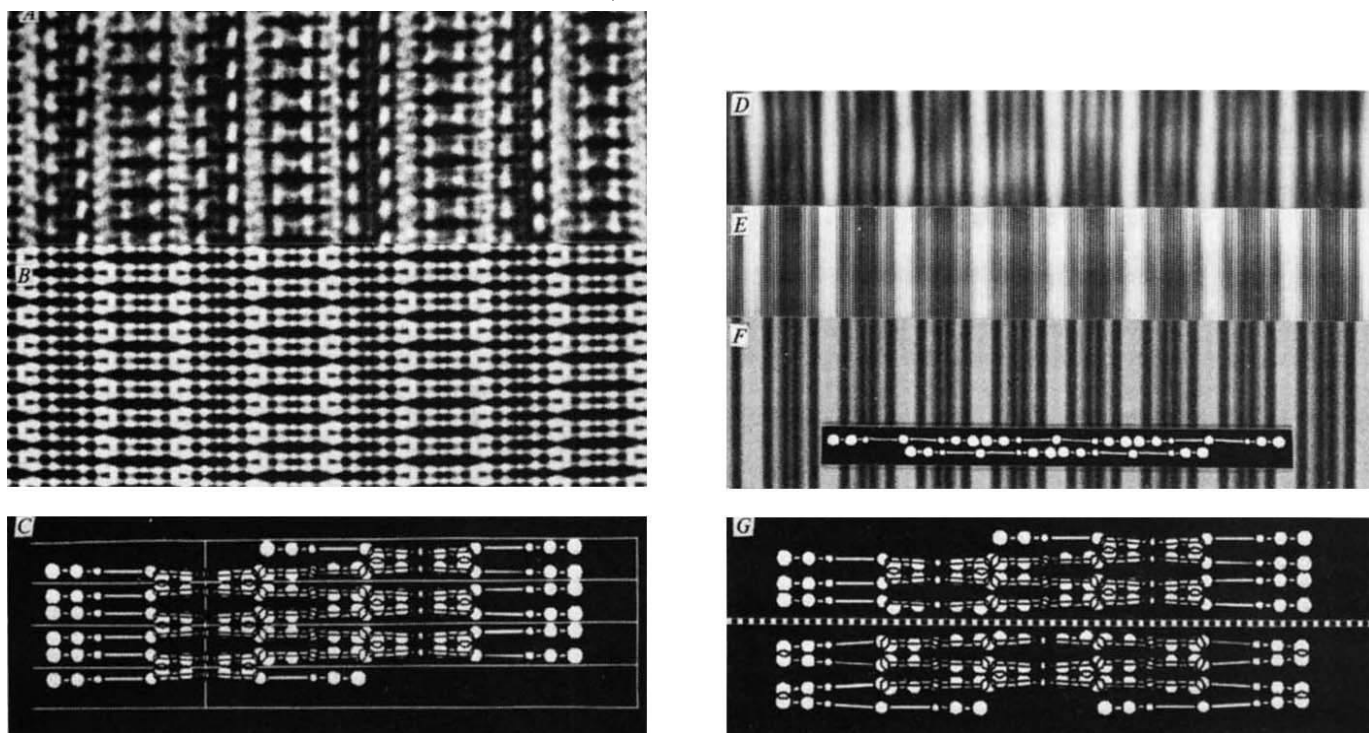


Fig. 2 Simulation of electron microscope images of the orthogonal sheet microcrystals and fibrin. *A*, Electron micrograph of a negatively contrasted microcrystal of the orthogonal sheet form²⁸; the image has been optically filtered to remove background noise^{44,45}. Unit cell dimensions are $900 \times 90 \text{ \AA}$. $\times 450,000$. *B*, TV display of computer simulation of *A*, based on the model below (*C*) (as described in the text). Note that the models in *C* and *G* are drawn to a scale about 1.5 times as great as the electron micrographs above. *D*, *E*, Computer filtered electron micrographs of negatively contrasted fibrin. The axial repeat is $225 \text{ \AA}$. In *D*, the fibrin is derived from native fibrinogen whereas in *E* the fibrin is from *Pseudomonas*-modified fibrinogen. Note that the weakest light band, which arises from the smallest globular domain, is less prominent in the modified fibrin^{26,28}, suggesting that the bacterial protease, like plasmin (see text), cleaves some material from this region. *F*, TV display of computer simulation of fibrin, based on the model shown in *G*. The fibrin band pattern was generated by superposition of arrays of the orthogonal sheet, shown in *A*, shifted by $225 \text{ \AA}$ (refs 26, 28). For clarity, two such layers of molecules are shown, separated by a dashed line, in the upper and lower portions of *G*. The lateral contacts in both fibrin and the orthogonal sheet form incorporate a half-stagger, where the central globular domain of one molecule fits into the 'pocket' formed by the end-to-end linkage of two molecules in an adjacent filament. A simple half-staggered array of five molecules is shown as an inset to *F* (and with the same scale) so that the relationship of the molecular shape and packing to the fibrin band pattern is apparent. The bright axial striations in both the orthogonal sheet form and fibrin arise from this juxtaposition of the end globular units of two molecules with the middle unit of a third. In the orthogonal sheet, the pairs of small bright units on either side of this feature can be seen to arise from the adjacent globular domains in these molecules. To account for the angle of the thin strands in the gap, the molecules are arrayed with a slight tilt. This arrangement generates the correct symmetry for the structure and also places the smallest globular units at the centre of the dark gap where they are seen as bright nodes.

sheet form, has indicated that all these structures seem to be made up of $450 \text{ \AA}$ -long fibrinogen molecules that bond end to end²⁸.

The best characterized macrocrystal form of fibrinogen is the monoclinic $P2_1$ crystal of protease-modified bovine fibrinogen^{27,28}. A packing model for this crystal was established using the morphology and special features of the diffraction patterns^{29,35}. Here too it was shown that the molecules are $450 \text{ \AA}$ long and bond end to end to form filaments. The general run of the molecules in the lattice was established but not their lateral relations. This knowledge of the molecular packing allowed an interpretation of electron microscope images of crystal fragments. In particular, a characteristic net or mesh form was observed that could be accounted for as the view along the unique symmetry axis (Fig. 3*A*, *D*). The strands of the mesh seen in the electron microscope were interpreted as pairs of symmetry-related molecular filaments in a distorted cell. These strands showed prominent features of macromolecular size, but again it was not possible using the Hall-Slayter model to define the lateral packing or to delineate individual molecules.

A form that we found useful in establishing the model was a 'segment' aggregate obtained from human fibrinogen modified by bacterial protease³⁶ (Fig. 4). The segments displayed a strikingly simple band pattern in negative staining that revealed where the molecules begin and end. Analysis of this structure showed that the molecules are in register in layers which are tilted alternately with respect to the long axis. Here again, the molecules were found to be $\sim 450 \text{ \AA}$ long.

Because there is no molecular overlap, the packing of fibrinogen in this form yields direct information about the mass

distribution along the molecule. The overall structure seems qualitatively like the Hall-Slayter model in that there are three major stain-excluding domains per molecular length. There is, however, additional fine structure in the segments. Within each major band there is another thin line of opposite contrast, which seems to define smaller domains. This evidence again suggested that the Hall-Slayter model was valid as a very low resolution image of the molecule, but that a more detailed mass distribution could be derived.

We may interpret the staining of the segments to mean that the outer domain of the Hall-Slayter model is, in fact, made up of two units, each about $30\text{--}40 \text{ \AA}$ in diameter. Close inspection of the segment images reveals an additional thin white line within the broad dark-staining region (Fig. 4). This feature indicates that another smaller globular unit may be located between the outer domain and the central nodule. This additional globular unit is, in fact, necessary to account for the detailed appearance of images of both fibrin and related forms as well as views of the crystal (see below). The molecule of length $450 \text{ \AA}$ thus appears in all the ordered aggregates as a heptad made up of seven globular domains. We show below how more precise dimensions of the globular units, as well as those of the rod-like regions connecting them, can be derived from the simulation of a variety of electron microscope images.

Simulation of electron microscope images

Using modifications of the model described above, computer simulations were carried out to develop a single molecular model whose substructure and packing could account for the negatively stained images of a variety of arrays seen in the

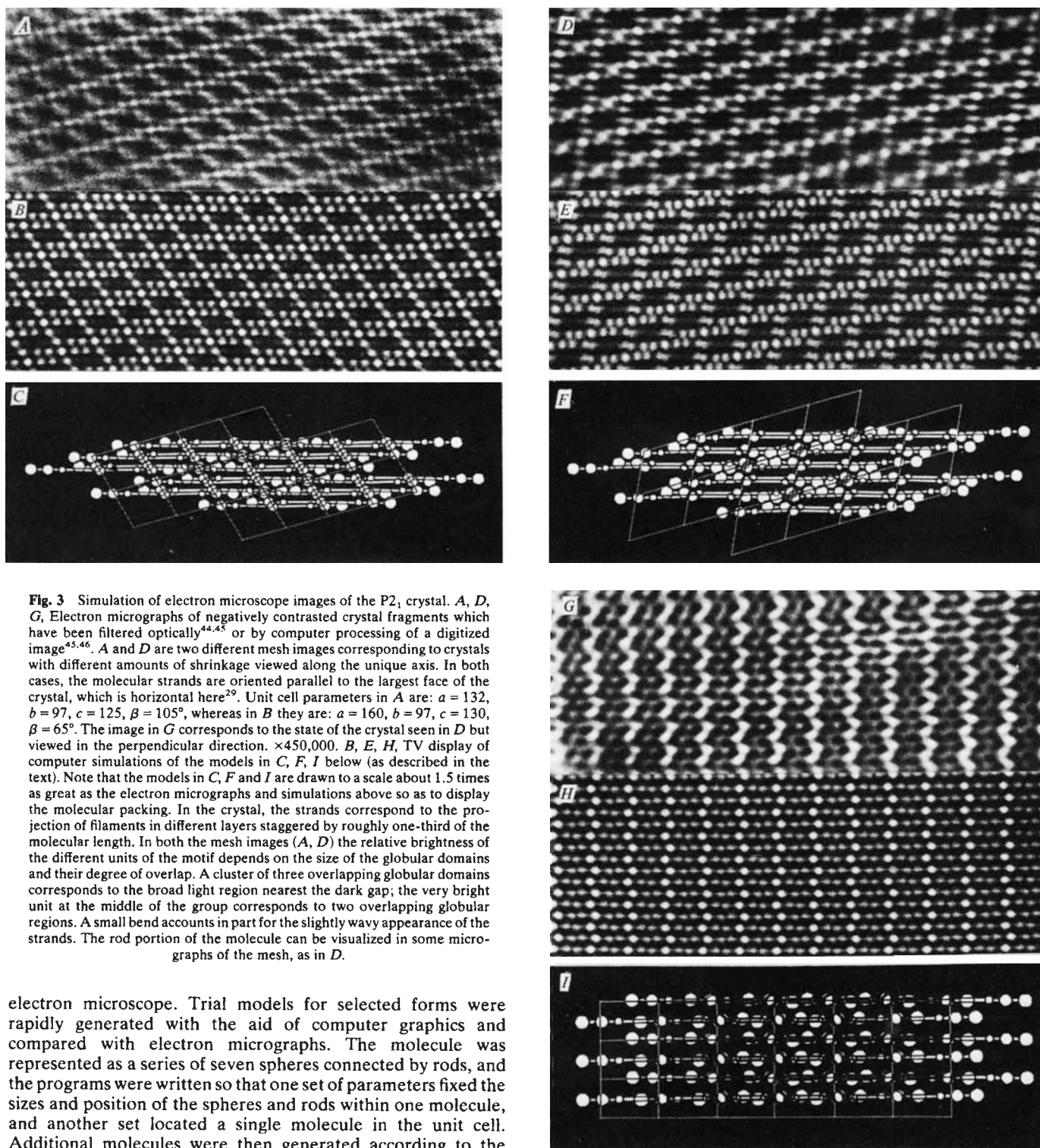


Fig. 3 Simulation of electron microscope images of the $P2_1$ crystal. *A, D, G*, Electron micrographs of negatively contrasted crystal fragments which have been filtered optically^{44,45} or by computer processing of a digitized image^{45,46}. *A* and *D* are two different mesh images corresponding to crystals with different amounts of shrinkage viewed along the unique axis. In both cases, the molecular strands are oriented parallel to the largest face of the crystal, which is horizontal here²⁹. Unit cell parameters in *A* are: $a = 132$, $b = 97$, $c = 125$, $\beta = 105^\circ$, whereas in *B* they are: $a = 160$, $b = 97$, $c = 130$, $\beta = 65^\circ$. The image in *G* corresponds to the state of the crystal seen in *D* but viewed in the perpendicular direction. $\times 450,000$. *B, E, H*, TV display of computer simulations of the models in *C, F, I* below (as described in the text). Note that the models in *C, F* and *I* are drawn to a scale about 1.5 times as great as the electron micrographs and simulations above so as to display the molecular packing. In the crystal, the strands correspond to the projection of filaments in different layers staggered by roughly one-third of the molecular length. In both the mesh images (*A, D*) the relative brightness of the different units of the motif depends on the size of the globular domains and their degree of overlap. A cluster of three overlapping globular domains corresponds to the broad light region nearest the dark gap; the very bright unit at the middle of the group corresponds to two overlapping globular regions. A small bend accounts in part for the slightly wavy appearance of the strands. The rod portion of the molecule can be visualized in some micrographs of the mesh, as in *D*.

electron microscope. Trial models for selected forms were rapidly generated with the aid of computer graphics and compared with electron micrographs. The molecule was represented as a series of seven spheres connected by rods, and the programs were written so that one set of parameters fixed the sizes and position of the spheres and rods within one molecule, and another set located a single molecule in the unit cell. Additional molecules were then generated according to the appropriate symmetry elements of the specific form under analysis. To convert these arrangements to density contours, which could then be compared with electron microscope images, the model was represented by sets of points spaced closely enough to correspond to a continuous structure at this resolution. The projected Fourier transform of this configuration was then computed, followed by the inverse transform which was displayed on a television screen. Subjective criteria were used to alter both molecular and packing parameters in comparing the simulation to filtered electron microscope images.

A range of molecular models was examined. Slight differences in the size of the globular units or slight departures of the two halves of the molecule from co-linearity were tested to achieve a good fit with a particular form. Some differences in the nature of the end-to-end molecular contacts were also tried with certain

arrays. It was found that all the images could be fitted with the same heptad model (Fig. 1*B*).

The $P2_1$ crystal

Several images from $P2_1$ crystal fragments could be simulated. The net described previously and identified as the view down the unique axis of the crystal is characterized by strands having features of macromolecular size running in the horizontal direction²⁹ (Fig. 3*A*). The repeating unit along the strands is a group of seven stain-excluding regions of varying sizes (none larger than ~ 40 Å)—the middle one being particularly bright—followed by a dark-staining gap. This motif repeats every 450 Å and corresponds to the projection of two symmetry-related

layers of molecular filaments in the unit cell. This form was particularly useful in establishing the size of the globular domains and their separation in the model. The small bend in the molecule accounts in part for the slightly wavy appearance of the strands. With a shift of about one-third the molecular length between filaments in the two layers the simulation shows excellent agreement with the filtered image of the electron micrograph (Fig. 3B, C).

Electron micrographs of the P₂₁ crystal fragments show a number of states of the mesh described above. Another commonly observed form has a mesh with a different appearance (Fig. 3D). The motif is rather similar to that described above, although the intensities and positions of the bright units differ somewhat. Keeping the parameters of the molecular model used in the previous simulation, only a slight change in the relative stagger of the molecular filaments within each layer is required for a satisfactory fit (Fig. 3E, F).

A different view of the crystal could also be identified. Some preparations contained very thin crystals that tended to lie with the large face on the grid. This image showed a distinctive pattern of alternating rows of bright and weak zig-zags repeated 145 Å axially (Fig. 3G). The spacings indicated that this was the view along one of the crystal axes perpendicular to the previous view and nearly perpendicular to the large face of the crystal (see Fig. 3D). Using the same arrangement of molecules as before (Fig. 3F), and simply taking the appropriate projection (Fig. 3I), a very satisfactory simulation can be achieved (Fig. 3H).

The orthogonal sheet form and fibrin

One of the most detailed images is that of the orthogonal sheet form, related to fibrin (Fig. 2A). Here the motif is a very complex dumbbell-shaped bright region about 400 Å long with a dark-staining gap of about 50 Å. The motifs in adjacent rows are staggered by half the lateral period so that the true axial repeat is 900 Å. Within the dark-staining gap, thin light strands seem to connect units in different rows. For the simulation, the dimensions of the molecular model as determined for the P₂₁ crystal views were maintained, but the molecular filaments in different layers were half-staggered, as determined previously^{13,26,28} (Fig. 2C). This arrangement accounts for the very bright axial striation which arises from juxtaposition of the end globular units of two molecules with the middle unit of a third (Fig. 2B). The small bright units on either side of this feature can be seen to arise from the adjacent globular domains in these molecules. To account for the angle of the thin strands in the gap, the molecules were arrayed with a slight tilt to the long axis of the unit cell. This arrangement generates the correct symmetry for the structure. The smallest globular units can be seen quite clearly as bright nodes at the centre of the dark gaps.

A half-stagger (225 Å) of this arrangement for the orthogonal sheet form then generates the fibrin image^{26,28} (Fig. 2E, F, G). The brightest band in fibrin again arises from juxtaposition of the end globular regions of two molecules together with a centre domain of a neighbouring molecule and the narrower band on either side from the adjacent domains. Additional features of the pattern are also accounted for: the thinnest band in the period corresponds to the smallest globular domain in the molecule. In fact, this arrangement is precisely that proposed in the early half-staggered models for fibrin¹³, but the size of the domains in the molecular model now yields correct dimensions for the band pattern (Fig. 2F inset). The relatively simple band pattern of fibrin is thus accounted for by a superposition of rather complex molecular arrays.

The molecular model

We have now obtained an improved low-resolution model for the fibrinogen molecule (Fig. 1B). The basic structure is closely related to the trinodular Hall-Slayer model, but we have been able to establish a precise length for the molecule of 450 Å, and a more detailed substructure. The outer globular domain, incompletely resolved by the shadowing technique used by Hall and Slayer, is now seen to consist of two regions of nearly equal

size. The narrow rod-like connection between the central and outer units had not previously been visualized, and we have shown that its regular run is interrupted by a small globular region displaced towards the outer domains. There is also a slight bending of the molecule by a few degrees. We consider the evidence in favour of this model to be compelling: all the features of the model are directly visible in certain electron microscope images, and the model can account for the detailed appearance of a variety of forms, including the complex pattern of fibrin.

Fibrinogen displays different properties at different stages of clotting. As shown for other fibrous proteins³⁷, particular domains may be associated with each of its various functions. The central and outer globular domains of the molecule are involved in assembly of the fibrin fibre; the middle region contains the small, highly charged fibrinopeptides cleaved by thrombin³⁸ whereas the outer globular domains contain complementary bonding sites for fibrin formation^{39,40}. Covalent cross-linking of the molecules by the fibrin-stabilizing factor has been shown to involve the outer globular domain⁴¹. Some of these sites may also be located in the neighbouring globular domain. The plasmin-sensitive portion of the molecule can be identified with the smallest globular domain which interrupts the regular run of the rod-like α -helical coiled-coil regions (Fig. 1B, D)^{23,24}. On the basis of this oversimplified picture, we can begin to localize a number of the characteristic properties of fibrinogen in different structural domains.

These results also indicate that a modest degree of molecular 'flexing' is a necessary feature of the packing arrangements in all the forms we have examined. Hence, the molecule should not be considered as rigid, but there is no compelling reason to postulate marked departures from colinearity of the two halves of the molecule.

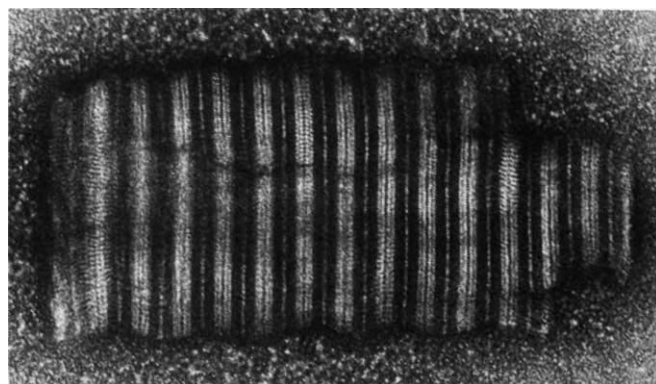


Fig. 4 Electron micrograph of negatively contrasted 'segment' prepared by precipitation of human fibrinogen modified by the *Pseudomonas* protease³⁶. The apparent axial repeat in the structure is 385 Å. The corrugations visible along the upper and lower edges indicate that the true period is 770 Å and that the molecules are both tilted in layers and bent. The end of the segment on the right shows where the molecules begin. $\times 135,000$.

The model described here represents the first stage in our approach to the detailed three-dimensional structure of fibrinogen. The dimensions used in our simulations were derived from negatively stained electron microscope images, but the approximate hydrated envelope can be calculated from the molecular volume in the crystal²⁹ (Fig. 1C). We have now completed three-dimensional X-ray data collection to about 20 Å on the P₂₁ crystal form. To derive truer shapes of the molecular domains we are refining the hydrated version of the model against the diffraction data⁴²; solvent flattening techniques may also be useful for phasing reflections at this resolution³⁰. The new model will reveal in even greater detail the complex architecture of this highly evolved protein molecule.

We thank Cynthia Stauffacher and Nancy Tooney for helpful discussions, Paul Norton for electron microscopy, William Saunders for photography, David DeRosier for computer pro-

grams and Edward Swinarski for computing. This work was supported by NIH grant AM17346 to C.C. and NSF grant PCM78-15913 to D.D. J.W.W. is an Established Investigator of the American Heart Association; G.N.P. held a National Research Service Award from the USPHS and is a Medical Foundation Fellow.

Received 4 July; accepted 4 November 1980.

1. Davie, E. W. & Fujikawa, T. A. *Rev. Biochem.* **44**, 799-829 (1975).
2. McFarlane, R. G. in *Human Blood Coagulation, Haemostasis and Thrombosis* (ed. Biggs, R.) 1-31 (Blackwell, Oxford, 1976).
3. Shulman, S. J. *Am. chem. Soc.* **75**, 5846-5852 (1953).
4. Caspary, E. A. & Kekwick, R. A. *Biochem. J.* **67**, 41-48 (1957).
5. Hall, C. E. & Slayter, H. S. *J. biophys. biochem. Cytol.* **5**, 11-15 (1959).
6. Köppl, G. *Nature* **212**, 1608-1609 (1966).
7. Krakow, W., Endres, G. F., Siegel, B. M. & Sheraga, H. A. *J. molec. Biol.* **71**, 95-103 (1972).
8. Bachmann, L., Schmitt-Fumian, W. W., Hammel, R. & Lederer, K. *Makromolec. Chem.* **176**, 2603-2618 (1975).
9. Marguerie, G. & Stuhmann, H. B. *J. molec. Biol.* **102**, 143-156 (1976).
10. Slayter, H. S. *Ultramicroscopy* **1**, 341-357 (1976).
11. Fowler, W. E. & Erickson, H. P. *J. molec. Biol.* **134**, 241-249 (1979).
12. Bailey, K., Astbury, W. T. & Rudall, K. M. *Nature* **151**, 716-717 (1943).
13. Stryer, L., Cohen, C. & Langridge, R. *Nature* **197**, 793-794 (1963).
14. Henschen, A. *Ark. Kemi* **22**, 355-373 (1964).
15. Henschen, A. *Thromb. Haemostasis* **42**, 14 (1979).
16. Blombäck, B., Hessel, B. & Hogg, D. *Thromb. Res.* **8**, 639-658 (1976).
17. Henschen, A. & Lottspeich, F. *Hoppe-Seyler's Z. physiol. Chem.* **357**, 1801-1803 (1976).
18. Doolittle, R. F. *Horizons Biochem. Biophys.* **3**, 164-191 (1977).
19. Lottspeich, F. & Henschen, A. *Hoppe-Seyler's Z. physiol. Chem.* **358**, 935-938 (1977).
20. Watt, K. W. K., Tagaki, T. & Doolittle, R. F. *Biochemistry* **18**, 68-76 (1979).

21. Doolittle, R. F., Watt, K. W. K., Cottrell, B. A., Strong, D. D. & Riley, M. *Nature* **280**, 464-468 (1979).
22. Henschen, A., Lottspeich, F. & Hessel, B. *Hoppe-Seyler's Z. physiol. Chem.* **360**, 1951-1956 (1979).
23. Doolittle, R. F., Goldbaum, D. M. & Doolittle, L. R. *J. molec. Biol.* **120**, 311-325 (1978).
24. Parry, D. A. D. *J. molec. Biol.* **120**, 545-551 (1978).
25. Cohen, C. *J. Polymer Sci.* **49**, 144-145 (1961).
26. Tooney, N. M. & Cohen, C. *Nature* **237**, 23-25 (1972).
27. Cohen, C. & Tooney, N. M. *Nature* **251**, 659-660 (1974).
28. Tooney, N. M. & Cohen, C. *J. molec. Biol.* **110**, 363-385 (1977).
29. Weisel, J. W., Warren, S. G. & Cohen, C. *J. molec. Biol.* **126**, 159-183 (1978).
30. Phillips, G. N., Lattman, E. E., Cummins, P., Lee, K. Y. & Cohen, C. *Nature* **278**, 413-417 (1979).
31. Cohen, C., Revel, J. P. & Kucera, J. *Science* **141**, 436-438 (1963).
32. Cohen, C., Slayter, H. S., Goldstein, L., Kucera, J. & Hall, C. *J. molec. Biol.* **22**, 385-388 (1966).
33. Kay, D. & Cuddigan, B. J. *Br. J. Haemat.* **13**, 341-347 (1967).
34. Karges, H. E. & Kuhn, K. *Eur. J. Biochem.* **14**, 94-97 (1970).
35. Tooney, N. M. & Weisel, J. W. in *Fibrous Proteins: Scientific, Industrial and Medical Aspects* Vol. 1 (eds Parry, D. A. D. & Creamer, L. K.) 393-427 (Academic, London, 1979).
36. Weisel, J. W., Tooney, N. M., Kaplan, I., Amrani, D. & Cohen, C. *J. molec. Biol.* **143**, 329-334 (1980).
37. Cohen, C. in *Molecular Architecture in Cell Physiology* (eds Hayashi, T. & Szent-Gyorgyi, A. G.) 169-190 (Prentice-Hall, New Jersey, 1966).
38. Blombäck, B. *et al. Nature* **218**, 130-134 (1968).
39. Blombäck, B., Hessel, B., Hogg, D. & Therkildsen, L. *Nature* **275**, 501-505 (1978).
40. Oleksa, S. A. & Budzynski, A. Z. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1374-1378 (1980).
41. Fowler, W. E., Erickson, H. P., Hantgan, R. R., McDonagh, J. & Hermans, J. *Science* (in the press).
42. Stauffacher, C., Phillips, G. N., Weisel, J. W., Ford, E. D. & Cohen, C. (in preparation).
43. Slayter, H. S. in *Principles and Techniques of Electron Microscopy* Vol. 9 (ed. Hayat, M.) 175-245 (Van Nostrand, New York, 1978).
44. Klug, A. & DeRosier, D. J. *Nature* **212**, 29-32 (1966).
45. Erickson, H. P., Voter, W. A. & Leonard, K. *Meth. Enzym.* **49**, 39-63 (1978).
46. Misell, D. L. in *Practical Methods in Electron Microscopy* Vol. 7 (ed. Glauret, A. M.) (North-Holland, Amsterdam, 1978).

LETTERS

Cosmic ray antiprotons and modified closed galaxy model

S. A. Stephens

Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400005, India

Observations suggest that a small flux of secondary antiprotons ($\bar{p}$), created in the interaction of cosmic ray nuclei with interstellar gas, should also be present in the primary cosmic radiation. Calculations based on accelerator data for the production of $\bar{p}$ and existing models for the propagation of cosmic rays, predict too small a flux of $\bar{p}$ compared with the finite flux observed recently¹. Although the observed excess could be attributed to the existence of primary cosmic ray antimatter, the experimental upper limits^{2,3} of the ratio of antinuclei to nuclei of charge $|Z| \geq 2$ seem to contradict this. Here we use a 'modified closed galaxy' model to attribute this excess to secondary antiprotons. In this model, ~50% of the observed cosmic ray nucleons are of recent origin and they propagate according to the 'nested leaky box' model, while the rest propagate according to 'closed galaxy' model. This model explains the observations on $\bar{p}$ and e^+ , and predicts more D and ^3He than do the existing models.

The secondary $\bar{p}$ spectrum in cosmic rays has been calculated in the past by Gaisser and Maurer⁴, and Badhwar *et al.*⁵ using the extensive accelerator data available for the production of $\bar{p}$. However, these calculated spectra differ by more than a factor of two at ~10 GeV and by even larger factors at lower energies, suggesting that cross-sectional representations used by them must have been responsible for this difference. The interpretation of a finite flux of $\bar{p}$ depends crucially on the accuracy of the estimated flux of $\bar{p}$ in cosmic rays. Szabelski *et al.*⁶ showed that the results of Badhwar *et al.*⁵ are in error. Thereby, they questioned the 'standard leaky box' model (SLBM) for the propagation of cosmic rays. I recently obtained a more accurate parametrization⁷ of the invariant cross-sections, for the production of $\bar{p}$ in inclusive reactions, and calculated the

production spectrum of $\bar{p}$. This calculation confirmed the conclusions of Szabelski *et al.*⁶. The results from this calculation⁷ are now used to examine the implication of the observed $\bar{p}$ in terms of cosmic ray propagation models.

The equilibrium spectrum of $\bar{p}$ has been obtained by taking into account ionization loss, annihilation of $\bar{p}$, and inelastic interactions in which $\bar{p}$ retains nearly half its energy. The resultant $\bar{p}/p$ ratio as a function of the kinetic energy of $\bar{p}$ is shown in Fig. 1 for various cosmic ray models. Curve S is the estimated $\bar{p}/p$ ratio according to SLBM, in which cosmic rays traverse 5 g cm^{-2} of interstellar gas independent of energy. This curve can be compared with other existing calculations⁴⁻⁶ carried out using SLBM only. The calculations of Gaisser and Maurer⁴, and Szabelski *et al.*⁶ agree within 25% with curve S over the entire energy region above 2 GeV. This small deviation arises partly from the differences in the parametrization of cross-sectional data and partly due to different demodulated proton spectra used. Badhwar *et al.*⁵ overestimate the $\bar{p}/p$ ratio by a factor of 2 at ~10 GeV and this difference increases with decreasing energy; however, they also agree within 25% at energies $\geq 50 \text{ GeV}$.

The SLBM has been abandoned since it was observed that the abundance ratio of secondary nuclei (like Li, Be and B) to primary nuclei (like C and O) decreased with increasing energy. This result suggested that high-energy cosmic rays traverse less amount of matter. Recent observations on the spectra of cosmic ray nuclei suggest⁸ that the energy dependence of the matter traversal is approximately proportional to $(8 \text{ GV}/c/R)^{1/2}$, where R is the rigidity in GV/c, for $R \geq 8 \text{ GV}/c$ and is nearly constant for $R < 8 \text{ GV}/c$. This dependence has been explained in two ways. (1) The confinement of cosmic rays in the Galaxy is energy dependent⁹—'modified leaky box' model (MLBM). (2) A certain fraction, f , of the matter traversed by cosmic rays in the source regions is energy dependent—'nested leaky box' model¹⁰ (NLBM). Curve M in Fig. 1 is the calculated $\bar{p}/p$ ratio on the basis of MLBM and the hatched region (N) is according to NLBM. The energy-dependent escape probability in NLBM from the source regions is assumed to be similar to that of MLBM, and that the median energy of interacting nucleons is ~10 times the energy of $\bar{p}$ produced; the upper and lower bounds are for $f = 0.5$ and 0.8 respectively. Figure 1 shows that

NLBM predicts too small a flux of $\bar{p}$ compared with MLBM in the energy region below a few tens of GeV.

Figure 1 plots the observed $\bar{p}/p$ ratio of $(5.2 \pm 1.5) \times 10^{-4}$ reported by Golden *et al.*¹ in the energy region 4.7–11.6 GeV; this result was based on 28 $\bar{p}$ events of extraterrestrial origin. The correct value may be higher by $\sim 15\%$ because the expected flux⁷ of $\bar{p}$ of atmospheric origin is smaller than that estimated by Golden *et al.* The $\bar{p}/p$ ratio obtained by Bogomolov *et al.*¹¹ is not plotted because it was based only on two $\bar{p}$ events, and in this experiment, splash albedo particles in the atmosphere could not be eliminated¹². Figure 1 shows that popular models of cosmic ray propagation predict too low a flux of $\bar{p}$ by a factor ≥ 4 compared with the observed flux; however, the significance of this difference is only about 3σ . In the following, we examine the 'closed galaxy' model originally proposed by Rasmussen and Peters¹³ to understand the apparently high flux of $\bar{p}$ observed by Golden *et al.*¹.

In the 'closed galaxy' model, cosmic rays are totally confined until they are destroyed by interaction. Curve C in Fig. 1 is the calculated $\bar{p}/p$ ratio on the basis of total confinement. The observed ratio falls short of curve C by a factor of ~ 2 . It is therefore proposed that $\sim 50\%$ of the observed cosmic ray nucleons are of recent ($\sim 10^7$ yr) origin, and are propagated according to either MLBM or NLBM; the remaining ones are the result of total confinement. We can now combine 50% of curve C with either curve M or hatched area N, and the resultant $\bar{p}/p$ ratio lies between the two short curves passing through the data point in Fig. 1. Thus, the 'modified closed galaxy' model (MCGM) can account for the observed flux of $\bar{p}$.

The secondary positron spectrum in cosmic rays can now be calculated using MCGM. The equilibrium spectrum of e^+ is

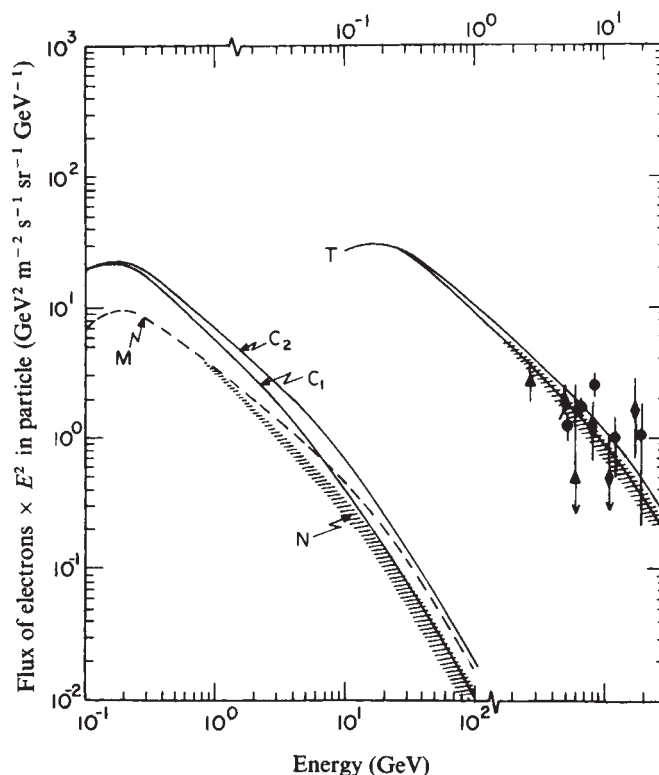


Fig. 2 The calculated energy spectra of the old and new components of secondary cosmic ray e^+ are shown in the left side, and the combined e^+ and total electron spectra on the right side; the flux values are multiplied by E^2 . Symbols as in Fig. 1; curves C_1 and C_2 correspond to $n_H = 0.1$ and 0.2 atom cm^{-3} , the mean hydrogen density of the interstellar gas traversed by the old component. Data are taken from: $\blacklozenge$, ref. 17; $\bullet$, ref. 18; $\blacktriangle$, ref. 19.

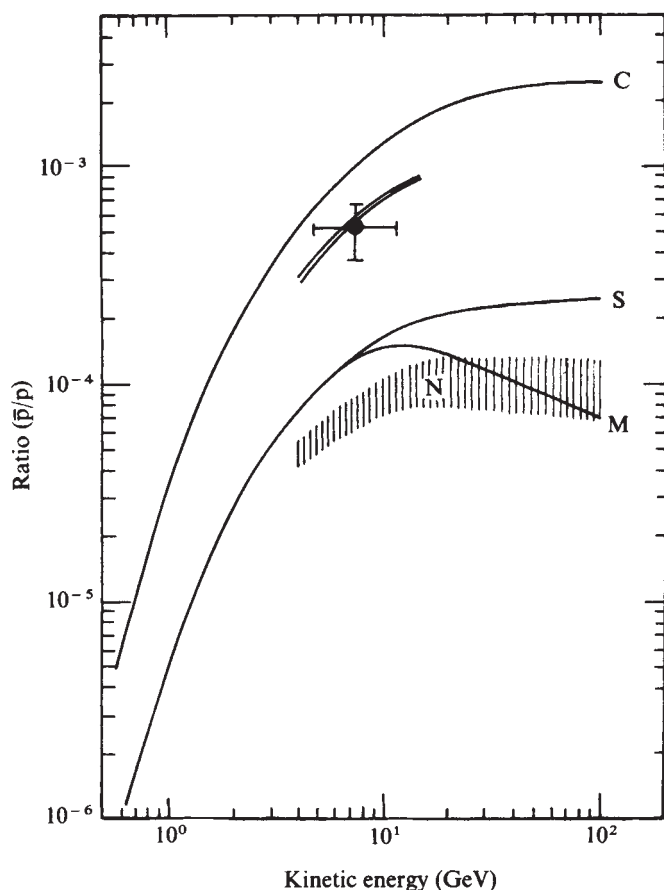


Fig. 1 The calculated $\bar{p}/p$ ratio plotted as a function of the kinetic energy of $\bar{p}$, for various models of cosmic ray propagation. The solid curves passing through the data point are the estimated ratio by summing 50% of curve C with 50% of either curve M or the lower bound of the hatched area N. $\bullet$, Data from ref. 1; S, SLBM; M, MLBM; N, NLBM; C, CGM.

calculated by using the recently estimated¹⁴ production spectrum and taking into account all energy-loss processes—ionization, bremsstrahlung, synchrotron and inverse Compton scattering. For convenience, the values of mean magnetic field B_1 and the radiation density are assumed to be the same for all models and are taken to be $4 \mu\text{G}$ and 0.7 eV cm^{-3} respectively.

The calculated spectra of e^+ from the old component are shown in Fig. 2 by curves C_1 and C_2 , which correspond respectively to $n_H = 0.1$ and 0.2 atom cm^{-3} , where n_H is the mean hydrogen density of the interstellar gas traversed by this component. The flux values in Fig. 2 are multiplied by E^2 . The flux of e^+ is proportional to n_H at high energies, where synchrotron and inverse Compton scattering losses dominate, and is independent of n_H at low energies, where ionization and bremsstrahlung losses dominate¹⁵. The dashed curve M is the calculated new component due to MLBM. In this calculation, the mean lifetime of cosmic rays of $R \leq 8 \text{ GV}/c$ is assumed to be 10^7 yr, as determined by radioisotope measurements¹⁶, and during this period they traverse 5 g cm^{-2} of interstellar gas. The recent e^+ component according to NLBM is calculated as in the case of $\bar{p}$ and is shown by the hatched area N.

The sum of the old and new components of the e^+ spectrum is shown on the right side in Fig. 2. The solid curves (T) are the sum of curves M and either C_1 or C_2 , and the hatched area corresponds to the sum of the hatched area N with curve C_1 or C_2 . The observed flux values of e^+ are plotted as filled data points at energies $\geq 3 \text{ GeV}$ (refs 17–19), where the effect due to solar modulation is small. The band representing MCGM agrees with the observations, which have a large scatter. Note that other existing models cannot satisfactorily explain e^+ observations^{6,12}. Also the value of n_H needed for the old component cannot be very much smaller than 0.1 atom cm^{-3} . This means that the effective confinement region for cosmic rays may not include regions very far away from the galactic disk, say larger than a few kpc. Also the total $(e^+ + e^-)$ spectrum in interstellar space

derived on the basis of curve T can be shown to be consistent with that deduced from the non-thermal radio spectrum in the Galaxy.

Half of the observed nucleons, belonging to the old component, is shared among various nuclear species approximately in proportion to the respective interaction mean-free paths. The estimated percentages of the old component among the observed p, He, (C + O), (F to Sc), (P to Cr) and (Fe Group) nuclei are respectively 58, 21, 9, 6, 4 and 3. Note that the new component constitutes >90% of the observed flux for nuclei other than p and He. Therefore, one can explain the observed energy dependence of the abundance ratio of secondary to primary nuclei using MLBM or NLBM for the new component. Further, the relative abundances of heavy nuclei of charge ≥ 6 would not differ from other models by more than a few per cent.

We now need to choose between MLBM and NLBM for the new component. According to MLBM, the energy spectrum of nucleons of recent origin above ≈ 8 GV/c (≈ 4 GeV/n) would be steeper than the injection spectrum by a power-law spectral index of about 0.5; the old component retains the injection spectrum. This would mean that the old component would dominate the observed flux beyond a certain energy²⁰. The calculated energies at which the intensities from these two components become comparable are ~ 56 , 460 and 1,950 GeV/n for He, (C + O) and (Fe group) respectively; protons have 58% old component even below 8 GeV. This clearly indicates that the observed similarity in the spectral shape of p and He above a few GeV/n cannot be understood, unless protons are accelerated differently. One should also expect a flattening of He spectrum beyond about 50 GeV/n, which has not been observed at least up to a few hundred GeV/n. These two inconsistencies discredit MLBM, and the natural choice is for NLBM, because the spectral shape of the new component is the same as the old.

A reliable estimate of the abundance of deuterium can be made using this model at relativistic energies. The estimated D/He ratio from this model is nearly twice as much from other models at energies ≤ 4 GeV/n and decreases slightly at high energies due to the energy-dependent confinement of the recent component in source regions. A similar enhancement is expected in the case of ^3He abundance. The available measurements have been restricted to energies ≤ 200 MeV/n and these results differ by a large factor²¹. However, they can be easily detected at relativistic energies using magnetic spectrometry²². The observation of an enhanced abundance of D would not only confirm the predictions of this model but would also reduce the difficulty in understanding the observed charge ratio of muons at sea level. Perhaps the distribution and spectral shape of high-energy γ rays, and the extended nonthermal radio emission in the Galaxy might also be explained by this model.

I thank Professors M. V. K. Apparao, R. R. Daniel and P. V. Ramana Murthy for helpful criticisms.

Received 15 September; accepted 10 November 1980.

1. Golden, R. L. *et al. Phys. Rev. Lett.* **43**, 1196–1199 (1979).
2. Badhwar, G. D. *et al. Nature* **274**, 137–139 (1978).
3. Smoot, G. F., Buffington, A. & Orth, C. D. *Phys. Rev. Lett.* **35**, 258–261 (1975).
4. Gaisser, T. K. & Maurer, R. H. *Phys. Rev. Lett.* **30**, 1264–1267 (1973).
5. Badhwar, G. D., Golden, R. L., Brown, M. L. & Lacy, J. L. *Astrophys. Space Sci.* **37**, 283–300 (1975).
6. Szabelski, J., Wdowczyk, J. & Wolfendale, A. W. *Nature* **285**, 386–388 (1980).
7. Stephens, S. A. *Astrophys. Space Sci.* (in the press).
8. Orth, C. D., Buffington, A., Smoot, G. F. & Mast, T. S. *Astrophys. J.* **226**, 1147–1161 (1978).
9. Juliusson, E., Mayer, P. & Muller, D. *Phys. Rev. Lett.* **29**, 445–448 (1972).
10. Cowsik, R. & Wilson, L. W. *Proc. 13th int. Cosmic Ray Conf.* **1**, 500–505 (1973).
11. Bogomolov, E. A. *et al. Proc. 16th int. Cosmic Ray Conf.* **1**, 330–335 (1979).
12. Daniel, R. R. & Stephens, S. A. *Bull. astr. Soc. Ind.* (in the press).
13. Rasmussen, I. L. & Peters, B. *Nature* **258**, 412–413 (1975).
14. Badhwar, G. D. & Stephens, S. A. *Proc. 15th int. Cosmic Ray Conf.* **1**, 398–403 (1977).
15. Badhwar, G. D. & Stephens, S. A. *Phys. Rev. D* **14**, 356–358 (1976).
16. Buffington, A., Orth, C. D. & Mast, T. S. *Astrophys. J.* **226**, 355–371 (1978).
17. Fanselow, J. L., Hartman, R. C., Hildebrand, R. H. & Mayer, P. *Astrophys. J.* **158**, 771–780 (1969).
18. Golden, R. L. *et al. Preprint PSL-PE00947* (1979).
19. Buffington, A. & Orth, C. D. *Astrophys. J.* **199**, 669–679 (1975).
20. Peters, B. & Westergaard, N. J. *Astrophys. Space Sci.* **48**, 21–46 (1977).
21. Daniel, R. R. & Stephens, S. A. *Space Science Review* **17**, 45–158 (1975).
22. Badhwar, G. D., Stephens, S. A. & Golden, R. L. *Proc. 14th int. Cosmic Ray Conf.* **9**, 3183–3187 (1975).

Expanding haloes in cometary comae

W.-H. Ip

Max-Planck-Institut für Aeronomie, D-3411 Katlenburg-Lindau 3, FRG

Observations indicate that cometary activity can be rather violent. Flare-ups, sunward-pointing jets and periodic formation of concentric haloes expanding outwards can also be persistent features in some comets. For example, a halo structure was observed in the coma of Comet Halley during its last visit¹. It is argued here that halo formation may be related to the propagation of shock pairs driven through the cometary atmosphere as a result of spin-modulation of the cometary outgassing rate. Even though the non-stationary process could be less dramatic than the example considered here, its effect on the chemistry and expansion of the neutral gas as well as dynamics of the cometary plasma must be significant.

Although theoretical models of cometary coma generally assume that neutral atmospheres expand spherically, symmetrically and without time variation, inhomogeneous structures have often been observed in the cometary comae. These include the formation of concentric shells (or haloes) moving outwards from the central source in succession and the appearance of fans or jets. Such non-stationary behaviour has long been thought to be related to the spin-modulation of the outgassing rate from the cometary nucleus^{2,3}.

There are two possibilities. If the surface composition of the cometary nucleus is uniform, the dependence of the surface temperature on the angle of insolation (θ) would mean higher gas emission rate at the point shifted from the sunlit position with $\theta = 0^\circ$. (The exact position of the 'hot spot' is determined by the direction and period of the nuclear spin as well as thermal properties of the surface material; the resultant jet effect has been used to explain the nongravitational acceleration or deceleration of comets².) As the jetting of the volatile gas—though localized—is more or less constant, the expansion of the cometary atmosphere, except the persistent sunward jets, should show no marked time variation. On the other hand, if the surface of the cometary nucleus can be divided into one active hemisphere and an inactive one (say), there will be more violent ejection of the volatile gas every time the active region turns towards the Sun. In the extreme case, there will be regular outbursts at the centre with a period equal to that of the spin period leading to large spatial and temporal variations in the structure of the coma. Clearly, the formation of expanding haloes as seen in the case of Comet Donati in 1858 (ref. 4) and Comet Halley¹ must be somehow related to such periodic outbursts. (From measurements of their respective halo structures, Whipple^{5,6} has determined the spin period of Comet Donati to be 4.6 h and for Comet Halley to be 10.3 h.)

To understand this phenomenon, time-dependent expansion of the gas outflow must be considered. Here it is assumed that the cometary atmosphere is an ideal gas and that the radial expansion is spherically symmetric. Furthermore, the time variation of the outgassing rate is approximated by a step function and we are basically interested in the initial propagation of the cometary neutral gas as the strong source is switched on. If ρ is the density, u the radial velocity, p the pressure and $\gamma (= 5/3)$ the ratio of specific heats of the gas, then

$$\frac{\partial \rho}{\partial t} + \frac{1}{r^2} \frac{\partial}{\partial r} (r^2 \rho u) = 0 \quad (1)$$

$$\frac{\partial u}{\partial t} + u \frac{\partial u}{\partial r} = -\frac{1}{\rho} \frac{\partial p}{\partial r} \quad (2)$$

$$\frac{\partial}{\partial t} \left(\frac{p}{\rho^\gamma} \right) + u \frac{\partial}{\partial r} \left(\frac{p}{\rho^\gamma} \right) = 0 \quad (3)$$

for the hydrodynamic equations of expansion of the neutral gas from a point source. Introducing the similarity variable⁷,

$$\eta = r/\alpha t \quad (4)$$

for the expansion of a piston gas with a constant speed α and defining

$$u = rV(\eta)/t, \quad \rho = \rho_0 r_0^2 \sigma(\eta)/r^2, \quad p = \rho_0 r_0^2 \pi(\eta)/t^2 \quad (5)$$

with the density distribution of the cometary coma at $t \leq 0$ given as:

$$\rho = \rho_0 r_0^2 / r^2 \quad (6)$$

(that is, $\rho = \rho_0$ at $r = r_0$ for the unperturbed flow), we can find an equivalent set of equations for V , σ and π with η as the sole variable. As discussed by Simon and Axford⁸, similarity solutions of V , σ and π can be obtained for the motion of such a piston gas describing a pair of shocks (S_+ and S_-) separated by a contact surface (C). The characteristics of the strong-shock solutions are that at the contact surface with $\eta \approx 0.84$, $V = 1$, $\pi = 0$ and $\sigma \rightarrow \infty$; and that the mass efflux from the source can be expressed as

$$Q = 4\pi f \alpha \rho_0 r_0^2 \quad (7)$$

where $f \approx 0.4$ in the case of strong shocks. With V , σ and π known for $\eta \geq 0$, the physical quantities u , ρ and p can be derived in turn for various values of r and t from equation (5).

Details of the similarity solutions for a blast wave can be found in ref. 8 and numerical solution of the above adiabatic non-steady flow equations in ref. 9. For brevity, only radial variation of the mass density will be discussed here. Figure 1 shows the profile of the gas density (ρ) plotted for the case of strong forward (S_+) and reverse (S_-) shocks. As $\sigma \rightarrow \infty$ at the contact surface (C), ρ increases to a large value also. For weaker shocks, the snow-plow effect of piling up the material between S_+ and S_- , though less pronounced, should be present. The similarity solutions of the piston gas apply only to the inner coma region which is still dominated by collisional effect. For a Halley-type comet with a nuclear radius (r_n) of 3 km and a surface number density (n_0) of $3 \times 10^{12} \text{ cm}^{-3}$, the nominal dimensions of such collision zone (r_c) is $\approx 1.4 \times 10^4 \text{ km}$. As a result of the varying outgassing rate, r_c could be a factor of 3 larger or one-third smaller as the comet rotates around. Even with the large value of $4 \times 10^4 \text{ km}$, r_c is still small compared with the radius of outer haloes ($\sim 3 \times 10^5 \text{ km}$) (see ref. 1). The compression effect by the piston gas must then stop a long way inside. However, the sharp definition of the haloes may still be maintained at large distances if the temperature of the gas at the contact surface is kept sufficiently low by collisional cooling. The delineation of the haloes is also helped by the rare-fraction region just behind the reverse-shock S_- . Indeed, the density variations as shown in Fig. 1 may explain why in some cases a dark zone is observed⁴ immediately behind a halo of enhanced brightness. Another effect is that the propagation speed of these haloes could be considerably lower than the value of 1 km s^{-1} generally quoted as the flow speed of the neutral gas in the inner coma close to the nucleus ($r \leq 10^{2-3} \text{ km}$) could be very small.

The dimension of these haloes as observed in the C_2 -emission are limited by the scale length of these molecules ($\approx 10^5 \text{ km}$ at 1 AU). Furthermore, the daughter fragments of the parent molecules (such as H_2O and CO) in the compressed shells would pick up extra kinetic energy during photodissociation or ion-molecular reactions such that the corresponding thin-shell structures would be dispersed. Therefore, expanding haloes are not likely to be seen in the density distributions of H, C and O atoms, for example. We may then envisage the formation of a new spherical halo moving outwards every time the active hemisphere of the cometary nucleus faces the Sun. Partly due to the pile-up of material between the forward and reverse shocks, and partly due to the projection effect along the line-of-sight, a concentric halo will be observed progressing outwards. Note that the time variation of the outgassing rate could be anything but a step function as adopted here and the shock velocity α

should have certain time variation. The dynamical process, however, is not likely to vary much as long as the active hemisphere of the comet can produce a strong driver gas. The real issue is, therefore, what is the energy source for such outbursts if they indeed are explosive.

In connection with the flare-ups of cometary activities sometimes observed, Donn and Urey¹⁰ have suggested that explosive chemical reactions involving free radicals or unstable molecules on the nuclear surface may provide the answer. The free-radical reactions studied in the laboratory are observed to be highly exothermic, yielding a heat of reaction of approximately a few electron volts per molecule. This would mean that there are pockets of unstable chemical mixture on the surface of the active hemisphere with energetic explosions triggered by solar radiation. Another possibility suggested by Greenberg¹¹ in the case of interstellar grains—is simply that some of the solid grains could release free energy stored in the mantle of organic molecules and free radicals through breakup of the cometary grains. Although highly uncertain, it is, nevertheless, interesting to look into the energy budget of the coma expansion. Taking the radial speed to be 1 km s^{-1} , the kinetic energy is then only of the order of 0.1 eV per H_2O molecule and about $\dot{E} \approx 10^{28} \text{ eV s}^{-1}$ in total if the gas production rate is $\approx 10^{29} \text{ H}_2\text{O molecules s}^{-1}$. An equivalent amount of chemical-energy source could be obtained if the sublimating ice contains 3% or so of the highly volatile material capable of exothermic chemical reaction. The energy budget of the halo expansion is therefore not a serious problem. In other words, even if the gas production rate were the same over one spin period, the contamination of one side with exothermic material would result in high-speed ejecta driving a shock pair periodically.

The hydrodynamics of the neutral coma in the inner region is not well understood. Various possible flow patterns including shock formation in steady-state conditions have been considered (see refs 12 and 13). The time-dependent expansion of the

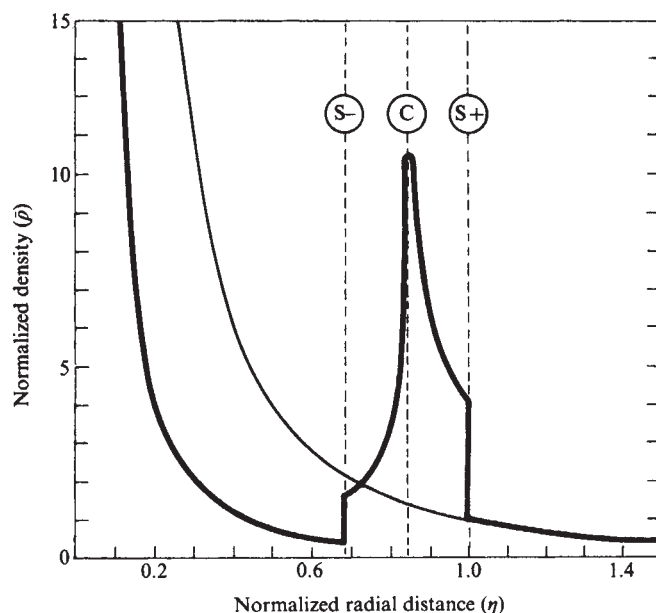


Fig. 1 The halo formation in the cometary comae through the compression effect of a piston shock. Radial variations of the density ($\bar{\rho}$) of the cometary neutral atmosphere normalized to the corresponding value at the position of the forward shock (S_+) at $\eta = 1$. Therefore $\bar{\rho} = \sigma(\eta)/\eta^2$ for $\eta = 1$ and $\bar{\rho} = 1/\eta^2$ for $\eta > 1$. The value of $\sigma(\eta)$ is obtained by taking a similarity solution to equations (1)–(6) with strong shocks (see ref. 8). The brightness enhancement in the haloes is caused by the density compression between the forward and reverse shocks. The dark zone sometimes observed immediately behind the haloes could be the manifestation of the rare-fraction region behind S_- as indicated. The density profile not modified by the shock pair is depicted by the thin curve.

cometary atmosphere should be even more complicated. Some idea can be derived from Bobrovnikoff's record of Comet Halley¹ where the expansion speed of the halo is estimated to be $\approx 0.25\text{--}0.5\text{ km s}^{-1}$. Identifying this value as the propagation speed of the contact surface and using the relation that the flow speed u^* behind the reverse shock should be larger by a factor of 2.5⁸, we obtain $u^* \approx 0.65\text{--}1.3\text{ km s}^{-1}$. This would mean that an additional energy source amounting to $\dot{E}^* \approx \frac{1}{2}Q(u^*)^2 \approx 10^{28}\text{ eV s}^{-1}$ (for $Q \approx 10^{29}\text{ H}_2\text{O molecules s}^{-1}$) is needed to drive the piston gas. This requirement can be accommodated by introducing a small fraction of exothermic volatile material into the sublimating gas.

Received 28 July; accepted 14 November 1980.

1. Bobrovnikoff, N. T. *Univ. Calif. Publ. Lick Obs.* **17**, 2 (1931).
2. Whipple, F. L. *Astrophys. J.* **111**, 375 (1950).
3. Sekanina, Z. *Icarus* **37**, 420 (1979).
4. Rahe, J., Donn, B. & Wurm, K. *Atlas of Cometary Forms* (NASA SP-198, 1969).
5. Whipple, F. L. *Nature* **272**, 134 (1978).
6. Whipple, F. L. *IAU Circ. No.* 3459 (1980).
7. Courant, R. & Friedrichs, K. O. *Supersonic Flow and Shock Waves* (Interscience, New York, 1948).

Whether any of these mechanisms may provide the energy required to drive a shock through the cometary atmosphere remains to be seen. But high-speed HCN jets ($u \geq 5\text{ km s}^{-1}$) have been detected in the coma of Comet Kohoutek (1973f) by Huebner *et al.*¹⁴, who also favoured explosive chemical reactions as the origin of these jets. Thus the formation of shock pairs together with a compression region in between must be considered a viable explanation for the expanding haloes. For illustration and simplicity we have considered only the strong-shock case for the similarity solutions. Other types of compression processes with weaker shocks must also exist; the present interpretation, nevertheless, highlights the nature of non-steady expansion of the cometary atmosphere.

8. Simon, M. & Axford, W. I. *Planet. Space Sci.* **14**, 901 (1966).
9. Hundhausen, A. J. *J. geophys. Res.* **78**, 1528 (1973).
10. Donn, B. & Urey, H. C. *Astrophys. J.* **123**, 339 (1956).
11. Greenberg, J. M. *Moon & Planets* **20**, 15 (1979).
12. Shul'man, L. M. in *Physics of Comets* (ed. Konopleva, V. P.) **84** (transl. NASA TT F-599, 1969).
13. Mendix, D. A. & Ip, W.-H. *Astrophys. Space Sci.* **39**, 335 (1976).
14. Huebner, W. F., Snyder, L. E. & Buhl, D. *Icarus* **23**, 580 (1974).

Blocking highs over Asia and monsoon droughts over India

C. R. V. Raman

MONEX, New Delhi-3, India

Y. P. Rao

B3/33 Azad Apartments, New Delhi-3, India

Severe summer droughts of the Indian subcontinent have been found to accompany prolonged 'breaks' in the south-west monsoon. Data from the years of severe drought suggest that the associated breaks were due to upper tropospheric blocking ridges over East Asia. We present here a model for the evolution of two such blocking ridges: the East Asia (EABR) and the West Asia (WABR). Apparently an intense WABR—the initiator of the monsoon break—precedes the formation of the EABR. This cycle could be a way of predicting years of intense drought or monsoon activity.

For a long time, studies of monsoon breaks were confined to changes in circulation in the monsoon area. Ramaswamy¹⁻³ was the first to associate breaks with the low index circulation in

middle latitude westerlies and the elongation of mid-tropospheric westerly troughs into India. However, eastward-moving westerly troughs penetrating into northern India enhance monsoon activity, and Ramanathan⁴ had visualized that migrating middle latitude ridges influence monsoon onset. Examining the severe droughts of 1979 (MONEX), 1972, 1966 and 1965, Raman *et al.*⁵ found that the breaks in these years were associated with stagnant upper tropospheric blocking ridges over the east Asia along $\sim 100^\circ\text{E}$, extending from 35°N to 70°N . In such situations, westerly troughs do not move beyond 80°E and instead tend to extend southwards.

Cold lows form south-west of the EABR and the lower tropospheric monsoon trough over India gets displaced northwards into the Himalayas—the most significant characteristic of breaks. We suggest that an upper tropospheric WABR along $\sim 50^\circ\text{E}$ is a necessary precursor to the development of the EABR. Also that in drought years, a well-marked surface high appears over USSR centred at 55°N and 50°E . This surface high is linked by a ridge with the Azores High over the central Atlantic. The surface high over the USSR, overlain by a ridge of up to 100 mbar, is due to the WABR. In contrast, in good monsoon years, the surface high and the associated upper ridge over the USSR are notably absent. Instead, a trough from the Aleutian low extends into Siberia with several low centres. Such a configuration is akin to the normal pattern.

Fig. 1 Evolution of the WABR and the EABR. Solid lines denote 200-mbar contours; H, high; L, low; dashed-dotted line indicates north-south orientation of the 200-mbar blocking ridge. Stage 1, Appearance of WABR. Stage 2, Maximum phase of WABR with intense cold lows on either side. Surface high delineated by pecked isobar. EABR had commenced formation a few days earlier. Lower tropospheric Monsoon Trough (MT) in normal position. Stage 3, EABR had intensified along with weakening of WABR. Low upstream of the EABR shifts southwards. Stage 4, Monsoon trough is displaced significantly northwards leading to monsoon break.

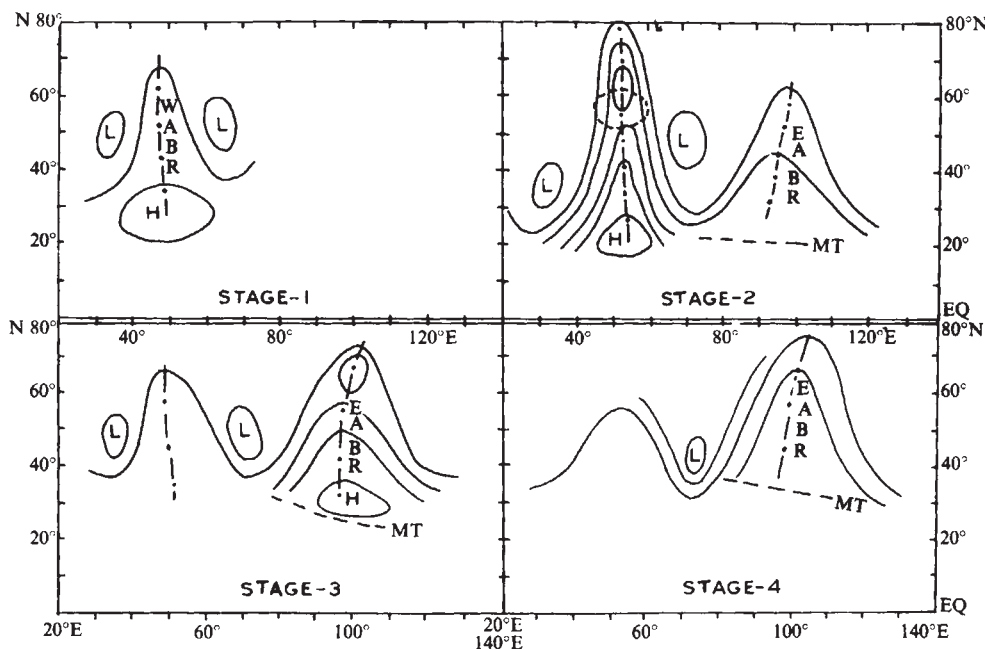


Table 1 Evolution of WABR and EABR

Month and year	Duration of monsoon break/ deficient monsoon rains	WABR (date of)			EABR (date of)		
		Appearance (mbar)	Max intensity with value of closed contour at 300 mbar/ 200 mbar/ (gpm)*	Formation of surface high with departure from normal (mbar)	Appearance (mbar)	Max intensity with value of closed contour at 300 mbar/ 200 mbar/ (gpm)*	Formation of surface high with departure from normal (mbar)
July 1972	18–31 July	4 July (200)	14 July (1,240)	14 July (+14)	8 July (200)	18 July (1,200)	25 July (+14)
August 1972	1–9 August	29 July (200)	4 August (1,208)	8 August (+15)	1 August (200)	5 August (1,216)	8 August (+15)
August 1966	23–27 August	12 August (300)	20 August (944)	23 August (+12.5)	18 August (300)	—	23 August (+23)
August 1965	4–15 August	25 July (300)	30 July (928)	4 August (+12.5)	31 July (300)	10 August (944)	10 August (+27)
July 1960	6–21 July	27 June (300)	1 July (944)	4 July (+12.5)	2 July (300)	5 July (952)	8 July (+20)

* gpm, Geopotential metres.

The evolution of the WABR and the EABR, during drought years from 1960, was obtained from Northern Hemispherical Synoptic charts⁶. The sequence of events preceding a monsoon break starts with the appearance of a predominant ridge at 200 mbar, first in the longitudinal belt 40–70°E, extending from the subtropical high pressure cell in the monsoon regime to almost 80°N. Stagnating at times for periods of up to one to two weeks, the WABR builds up as a warm high, reaching almost to the surface in a week after appearance. At its intense phase, even a closed high develops in the whole column, with the surface pressure departing from the long term normal (1931–60) by as much as +20 mbar. Such a deep meridionally-oriented blocking ridge configuration over west Asia elongates the westerly troughs both on the upstream (west European) and the

downstream (central Asian) sides. About a week after the maximum phase, the WABR begins to weaken.

The EABR generally appears in the belt 90–120°E some four days after the WABR forms, and extends from 35 to 70°N. The EABR begins to build up in a similar way to the WABR, generally when the latter starts to shrink in meridional extent and weaken. During this phase of the build up of the EABR, the westerly trough over central Asia between the WABR and the EABR further elongates to more southern latitudes. (The temperatures in this trough are ~5–8°C below normal in the mid-troposphere but warmer than the surrounding at 200 mbar and 100 mbar.) The superposition of this elongated cold upper trough/low at southern latitudes is manifested as the displace-

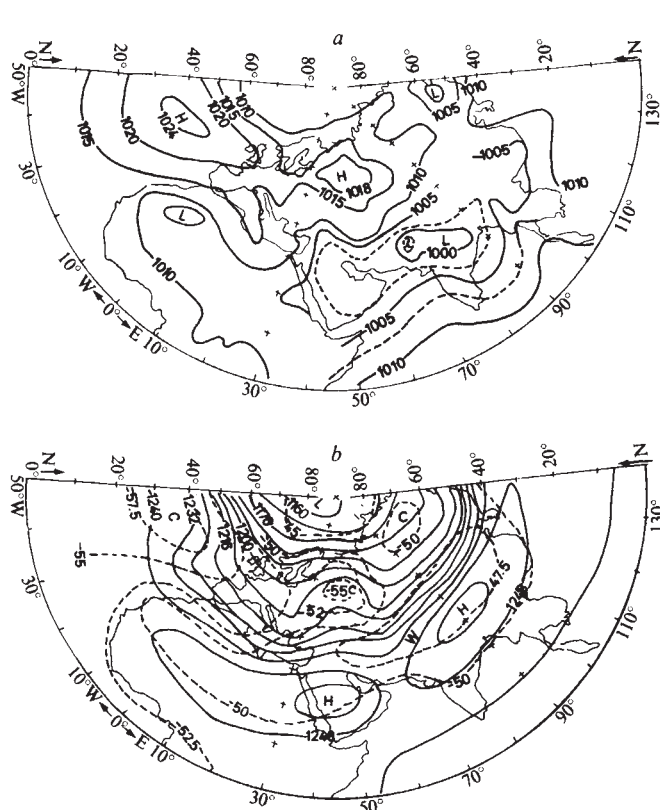


Fig. 2 Mean conditions in August 1972, a year of severe monsoon drought. a, Surface isobars. Note marked high centre at 55°N 50°E and the associated ridge linking with the Azores High over the central Atlantic. The ridge extends to 90°E across Eurasia. b, 200 mbar. Solid lines denote contours and dashed lines represent temperature (°C). C, cold; W, warm. Note markedly cold upper tropospheric ridge along 50°E, above the surface high over USSR.

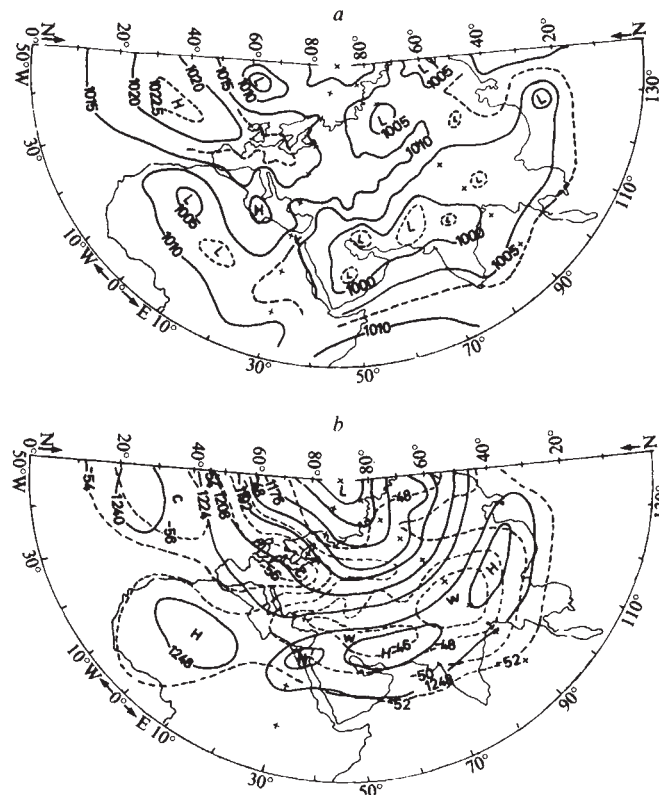


Fig. 3 Mean conditions during August 1975, a year of good monsoon rains. a, Surface isobars. Note the absence of a high centre over the USSR. Instead, a trough from the Aleutian low extends into Siberia with several low centres. Note that the monsoon low over India and neighbourhood is more intense than in Fig. 2a (1972). b, 200 mbar. Solid lines denote contours and dashed lines represent temperature (°C). Note the circumpolar flow and absence of ridges along 50°E.

ment of the lower tropospheric monsoon trough (MT) to the north into the Himalayas—symptomatic of the monsoon break. The build-up of an intense WABR seems essential for creating the EABR, which brings about the monsoon break. This cycle of the WABR building up well before the break could be useful in predicting monsoon breaks and consequent droughts. The sequence of evolution is presented in Fig. 1. A few examples of the cycle are given in Table 1.

Certain broad features of the WABR and the EABR are striking even in the monthly mean charts of 1972, a severe drought year. Figures 2 and 3 present the surface and upper air circulation features during August 1972 and 1975 respectively. In 1972, the WABR had started to build-up by the beginning of June and continued during the first half of July 1972. The subsequent build up of the EABR coincided with the pronounced break over India during the second half of July. The WABR/EABR configuration continued in August 1972, when the monsoon rains failed. The WABR prominent in Fig. 2b at 200 mbar, is associated with cold temperatures along $\sim 50^\circ\text{E}$. The surface high vertically below this 200 mbar ridge is quite prominent (Fig. 2a). The EABR is less marked in the monthly mean chart (Fig. 2b), although it is seen markedly in decadal mean charts covering break occurrences in this and other years.

In the good monsoon year of 1975, the circulation at 200 mbar (Fig. 3b) is more circumpolar with a weak trough in the location of the 1972 WABR (50°E). The contrasts between the good and bad monsoon years described above in the surface pressure distribution and the upper air features from the eastern Atlantic to east Asia are shown by other years during the past two decades.

Negative temperature anomalies are noticed at 200 mbar and above in WABR and EABR, with positive anomalies in the troposphere. The tropospheric warmth seems to be associated with the subsidence characteristic of high pressure. The stratospheric cooling must be due to the ascent, and maintains the high. Hoskins and Simmons have revived interest in downstream development as an important atmospheric scale phenomenon. It might be useful to apply this study to monsoon variability over India.

Received 4 August; accepted 14 November 1980.

1. Ramaswamy, C. *Geophysica* **6**, 455–477 (1958).
2. Ramaswamy, C. *Tellus* **14**, 337–349 (1962).
3. Ramaswamy, C. *Bull. Ind. natn. Sci. Acad.* **54**, 109–132 (1975–76).
4. Ramanathan, A. S. *J. mar. Biol. Ass. Ind.* **14**, 843–861 (1972).
5. Raman, C. R. V., Rao, Y. P. & Alvi, S. M. A. *Curr. Sci. Ind.* **49**, 123–129 (1980).
6. *Synoptic Bull. Northern Hemisphere Parts I–III* (USSR, 1960–1978).
7. Simmons, A. J. & Hoskins, B. J. *J. atmos. Sci.* **36**, 1239–1254 (1979).

Possible role for metals in stratospheric chlorine chemistry

Edmond Murad & William Swider

Air Force Geophysics Laboratory, Hanscom Air Force Base, Massachusetts 01731

Sidney W. Benson

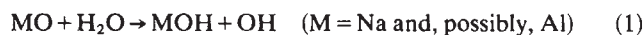
Department of Chemistry, University of Southern California, Los Angeles, California 90007

Meteoroids ablate largely in the Earth's ionosphere (at altitudes between 80 and 120 km) and the products are seen mostly as atomic ions^{1–3}. Atomic neutral atoms have also been observed and studied optically, but the data are not extensive for elements other than sodium^{4,5}. The processes responsible for maintaining the equilibrium of metallic atoms and ions in the ionosphere are understood generally^{6–9}. Below 80 km, the metal ions are largely neutralized. The chemistry which occurs during the subsequent transport of the metals to the ground is uncertain; aerosols collected in the lower stratosphere have been reported to contain chlorides and sulphates of metals (some of which occur in meteors)^{10–12} while aerosols collected in the troposphere have been found to contain many metals among which are meteor metals^{13–16}. It has been suggested that ions of the type $\text{Na}^+ \cdot (\text{H}_2\text{O})_n$ may be present in the upper stratosphere¹⁷, their presence being plausible in view of a meteor sodium model which indicated that large amounts of NaOH (almost 10^6 cm^{-3}) would be present at 40 km (ref. 18). This latter conclusion was questioned¹⁹ in view of thermochemical data which indicated that NaOH, once formed, should be removed quickly by reaction with Cl, ClO and HCl. We consider here reactions of meteor, and other, metals with Cl, ClO and HCl to assess the impact of halocarbons on ozone. Release of 1,000 tonne of sodium (or similar metal) may significantly reduce the harmful effects of halocarbons on ozone for several years.

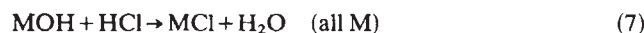
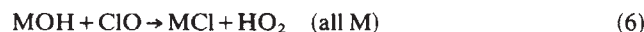
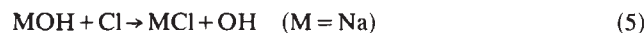
Although sodium comprises only about 2% by weight of the total metal concentration in meteors²⁰, its reaction with Cl, ClO and HCl may represent a class of reactions which could be important in the chemical maintenance of these species in the stratosphere (the condensation of Ga, Ge and Sb in nebulae has been suggested to proceed through the respective metal hydroxide and metal chloride intermediates²¹). The other major

metallic constituents of meteors are (by weight): Al(1.7%), Ca(1%), Fe(11.5%), Mg(12.5%), Ni(1.5%) and Si(20%). Here Si is included in the term 'metallics'. Other, less abundant metals (such as Ti, Cr, Mn) have been observed in micrometeorites collected in the stratosphere²².

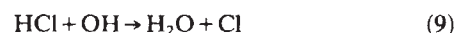
We now consider chemical reactions involving these metals or their intermediates with Cl, ClO and HCl, even though a quantitative evaluation of these reactions is not possible because of the lack of kinetic and thermochemical data. Cl, ClO and HCl have significant concentrations at altitudes between 15 and 50 km (refs 23, 24). Sodium will probably be in the form of NaOH (ref. 18) and/or NaHCO_3 by the time it has descended to 50 km (refs 19, 25). Similar compounds will be formed in the case of the other metals. The basic mechanism for the formation of the metal hydroxides (MOH) and the bicarbonates (MHCO_3) seems to be:



where M is any meteor metal and X is any third-body. Metal chlorides, MCl, may then be formed by the reactions:



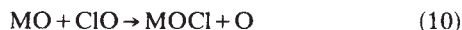
Reactions (7) and (8) are expected to proceed with large rate coefficients ($\sim 10^{-9} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$) and can deplete HCl significantly relative to the reaction



which is an important step in the chain reaction leading to the depletion of ozone²⁶. Reaction (9) has a rate coefficient²⁷ of $6.6 \times 10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ so that even if $k_7 + k_8$ [rate coefficients for reactions (7) and (8)] were a factor of 1,000 less than estimated (that is $\sim 10^{-12} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$), they would still be important competition for reaction (9) for $[\text{OH}] \sim [\text{MOH}] + [\text{MHCO}_3]$; the data of refs 18 and 23 indicate this to be true at $\sim 20 \text{ km}$. Of the meteor metals, sodium and aluminium

can readily form chlorides through reactions (7) and (8). The possibility of forming hydroxides of other metals (Fe, Mg and Ca) by three-body reactions or by reactions of the monoxides with minor constituents (such as HO_2) should not be discounted.

Silicon is likely to enter the stratosphere as SiO_2 as the large dissociation energy of the monoxide, $D_0^\circ(\text{Si-O}) = 190 \pm 2 \text{ kcal mol}^{-1}$ (ref. 28), precludes its reduction by O atoms. SiO_2 might also form weakly bonded compounds with Cl, ClO and HCl; photochemical studies indicate that solid SiO_2 catalyses the rate of photochemical decomposition of halocarbons²⁹. Reactions leading to the formation of MOCl may also be possible, perhaps through



Thermochemical data are available only for AlOCl , and these indicate that the dissociation energy of AlOCl , $D_0^\circ(\text{AlO-Cl})$ is greater than $D_0^\circ(\text{Cl-O})$ or $D_0^\circ(\text{H-Cl})$ (ref. 30). Indeed, analysis of the high-temperature reactions among the constituents of solid-fuel rocket engines predicts large amounts of AlOH and AlOCl at the expense of Al_2O_3 and HCl (ref. 31) (note that the engine of the space shuttle releases considerable amounts of Al_2O_3 , Fe and FeCl_2 (ref. 32)).

A quantitative assessment of the importance of reactions (1)–(8) will have to await the availability of their rate coefficients. However, the strongly ionic character of many of these metals suggests that reactions of the above type will be fast. For example, Ca and ClO_2 react to yield both CaO and CaCl and the total reaction cross-section has been estimated to be 10^{-14} cm^2 at thermal energies³³, which corresponds to a rate coefficient of $10^{-9} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$. Taking $k_7 + k_8$ to be $10^{-11} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ (which is a conservative estimate) and using $[\text{MHC}_3] + [\text{MOH}] \sim 2 \times 10^6 \text{ cm}^{-3}$ for $\text{M} = \text{Al} + \text{Na}$ at 40 km [this is equivalent to twice the calculated total sodium compound concentration at 40 km (ref. 18)], then the time constant for reactions 7 + 8 is $5 \times 10^4 \text{ s}$. On the other hand, reaction (9), the dominant process for the removal of HCl not involving metals, has a time constant of $\sim 2 \times 10^6 \text{ s}$ because $[\text{OH}] \sim 10^6 \text{ cm}^{-3}$ and $k_9 = 6.6 \times 10^{-13} \text{ cm}^3 \text{ molecule}^{-1} \text{ s}^{-1}$ (ref. 27). This shows that reactions such as (7) and (8) may be important.

The metal chlorides, particularly NaCl , are very stable in the stratosphere, and would be expected to act as nucleating agents, eventually being incorporated in aerosols. (Rocket exhaust which contains metals such as aluminium has been found to enhance ice nucleation³⁴.) Observations indicate the presence of Cl (refs 10–12, 16, 35), and Na in some aerosols. At high temperatures both condensed and gaseous Na_2SO_4 , a possible aerosol constituent, reacts^{36,37} with HCl to yield NaCl . NaCl will, of course, polymerize readily. The condensation of NaCl will probably be in the form of heterogeneous nucleation of monomer, dimer or trimer NaCl with other constituents such as water molecules or sulphur dioxide. A cluster containing 100 molecules, only 1–3 of which are NaCl , will have a concentration of $\sim 10^6 \text{ cm}^{-3}$ and its sodium would be undetectable. On the other hand, a cluster of NaCl , $(\text{NaCl})_n$, with $n = 100$, would have a concentration of $\sim 10^4 \text{ cm}^{-3}$ and would again be undetectable in the atmosphere.

How much metal is deposited by meteors and how does this compare with the total amount of halocarbons deposited in the atmosphere? Parkin and Tilles³⁸ estimated the total meteor influx to the Earth to be from $3.6 \times 10^{10} \text{ g yr}^{-1}$ to $3.6 \times 10^{11} \text{ g yr}^{-1}$. Gadsden⁷ estimated the total meteor influx to be $9 \times 10^{10} \text{ g yr}^{-1}$ ($9 \times 10^4 \text{ tonnes per yr}$), corresponding to a total metal atom influx of $2 \times 10^6 \text{ metal atoms cm}^{-2} \text{ s}^{-1}$. Hughes³⁹ quoted values in the range 3×10^8 – $5 \times 10^{13} \text{ g yr}^{-1}$ with a recommended estimate of $5 \times 10^8 \text{ g yr}^{-1}$. Later, Hughes⁴⁰ reviewed the data and estimated the total amount of meteors deposited into the Earth to be $1.6 \times 10^{10} \text{ g yr}^{-1}$. Hunten *et al.*⁴¹ analysed this problem from the point of view of smoke and dust particles deposited in the Earth's mesosphere and stratosphere by meteoroids and preferred the value given by Hughes⁴⁰,

although the possibility of a higher value was noted, and suggested⁴¹ a meteoric origin for the condensation nuclei of sulphate aerosols. The total weight of halocarbons deposited in the atmosphere has been estimated to be $\sim 7 \times 10^5 \text{ tonnes yr}^{-1}$. If Gadsden's estimate⁷ is correct, then meteor metals may be important in the chemistry and fate of the halocarbons in the stratosphere.

High-flying aircraft provide two additional sources of metals in the stratosphere through the combustion of jet fuels and the erosion of engine parts (the latter being orders of magnitude greater than the former⁴²). The principal metal contaminants in the fuels are iron, copper, calcium, vanadium and sodium, and these may be released in amounts varying between 100 and 1,000 tonnes yr^{-1} depending on the type of fuel and on the frequency of flights⁴³. Engine erosion elements include predominantly aluminium, manganese and chromium.

An intentional or an unintentional release (such as, jet fuels and engine erosion) in the stratosphere of $\sim 10^3 \text{ tonnes}$ of Na and, possibly, Al might reduce $[\text{Cl}]$ and $[\text{ClO}]$ by a factor of two. It would require several years for the diffusion cycle to re-establish those concentrations if the release occurred above 30 km. Finally, although the above discussion is speculative, it may help explain recent controversial results obtained by Anderson *et al.*⁴⁴, who were the first to measure $[\text{Cl}]$, $[\text{ClO}]$ and $[\text{O}_3]$ simultaneously. Their results indicate some difficulties in current chemical models used to predict the effect of these species on $[\text{O}_3]$.

We thank Dr R. S. Narcisi for helpful comments.

Received 16 April; accepted 15 November 1980.

- Narcisi, R. S. *Space Res.* **8**, 360–369 (1968).
- Zbinden, P. A., Hidalgo, M. A., Eberhardt, P. & Geiss, J. *Planet. Space Sci.* **23**, 1621–1642 (1975).
- Goldberg, R. A. & Aikin, A. C. *Science* **180**, 294–296 (1973).
- Hunten, D. M. *Space Sci. Rev.* **6**, 493–573 (1967).
- Megie, G., Bos, F., Blamont, J. E. & Chanin, M. L. *Planet. Space Sci.* **26**, 27–35 (1978).
- Swider, W. *Planet. Space Sci.* **17**, 1233–1246 (1969).
- Gadsden, M. *Annls Geophys.* **26**, 141–150 (1970).
- Brown, T. L. *Chem. Rev.* **73**, 645–667 (1973).
- Murad, E. *J. geophys. Res.* **83**, 5525–5530 (1978).
- Shedlovsky, J. P. & Paisley, S. *Tellus* **18**, 499–503 (1966).
- Delaney, A. C., Shedlovsky, J. P. & Pollock, W. H. *J. geophys. Res.* **79**, 5646–5650 (1974).
- Lazrus, A. L. & Gandrud, B. *J. geophys. Res.* **79**, 3424–3431 (1971).
- Cadle, R. D. & Grams, G. W. *Rev. Geophys. Space Phys.* **13**, 475–501 (1975).
- Charlson, R. J., Covert, D. S., Larson, T. V. & Waggoner, A. P. *Atmos. Envir.* **12**, 39–53 (1978).
- Penkett, S. A., Atkins, D. H. & Unsworth, M. H. *Tellus* **31**, 295–307 (1979).
- Parungo, F., Ackerman, E., Caldwell, W. & Weickmann, H. K. *Tellus* **13**, 521–529 (1979).
- Ferguson, E. E. *Geophys. Res. Lett.* **5**, 1035–1038 (1978).
- Liu, S. C. & Reid, G. C. *Geophys. Res. Lett.* **6**, 283–286 (1979).
- Murad, E. & Swider, W. *Geophys. Res. Lett.* **6**, 929–932 (1979).
- Dube, A. *et al.* in *Mineral Science Investigations, 1974–75* (ed. Mason, B.) (Smithsonian Contributions to the Earth Sciences No. 19, Washington DC, 1975).
- Wai, C. M. & Wasson, J. T. *Nature* **282**, 790–793 (1979).
- Ganapathy, R. & Brownlee, D. E. *Science* **206**, 1075–1077 (1979).
- Logan, J. A., Prather, M. J., Wofsy, S. C. & McElroy, M. B. *Phil. Trans. R. Soc.* **290**, 187–234 (1978).
- Abbas, M. M. *et al.* *J. geophys. Res.* **84**, 2681–2690 (1979).
- Keneshea, T. J., Murad, E., Swider, W. & Zimmermann, S. P. *EOS* **60**, 898 (1979).
- Rowland, F. S. & Molina, M. J. *Rev. Geophys. Space Phys.* **13**, 1–35 (1975).
- Baulch, D. L. *et al.* *J. phys. Chem. Ref. Data* **9**, 295–472 (1980).
- Stull, D. R. & Prophet, H. (eds) *JANAF Thermochemical Tables 2nd edn* NSRDS-NBS-37 (US Government Printing Office, Washington DC, 1971).
- Ausloos, P., Rebber, R. E. & Glasgow, L. J. *Res. natn. Bur. Sids*, **82**, 1–8 (1977).
- Chase, Jr., M. W., Curnutt, J. L., McDonald, R. A. & Syverud, A. N. *J. phys. Chem. Ref. Data* **7**, 311–480 (1978).
- Park, C. *Atmos. Envir.* **10**, 693–702 (1976).
- Isaacs, R. G., Burke, H.-H. & Sze, N. D. *Environmental Impact Studies for Several Space Transportation Vehicles* (Environmental Research and Technology, No. P-3139, Concord, Massachusetts, 1977).
- Engelke, F., Sander, R. K. & Zare, R. N. *J. chem. Phys.* **65**, 1146–1155 (1976).
- Parungo, F. P. & Allee, P. A. *J. appl. Met.* **17**, 1856–1863 (1978).
- Lazrus, A. L., Gandrud, B. & Cadle, R. D. *J. geophys. Res.* **76**, 8083–8088 (1971).
- Stearns, C. A., Kohl, F. J., Fryburg, G. C. & Miller, R. A. in *High Temperature Metal Halide Chemistry* (eds Hildenbrand, D. L. & Cubicciotti, D. D.), 555–573 (Electrochemical Society, Princeton, 1977).
- Hancock, P. in *High Temperature Metal Halide Chemistry* (eds Hildenbrand, D. L. & Cubicciotti, D. D.), 645–669 (Electrochemical Society, Princeton, 1977).
- Parkin, D. W. & Tilles, D. *Science* **159**, 936–946 (1968).
- Hughes, D. W. *Planet. Space Sci.* **20**, 1949–1959 (1972).
- Hughes, D. W. *Space Res.* **15**, 531–539 (1975).
- Hunten, D. M., Turco, R. P. & Toon, O. B. *J. atmos. Sci.* **37**, 1342–1357 (1980).
- CIAP Monogr. 2, *Propulsion Effluents in the Stratosphere*, DOT-TST-75-52 (NTIS, Springfield, 1975).
- CIAP Monogr. 3, *The Stratosphere Perturbed by Propulsion Effluents*, DOT-TST-75-53 (NTIS, Springfield, 1975).
- Anderson, J. G., Grassl, H. J., Shetter, R. E. & Margitan, J. J. *geophys. Res.* **85**, 2869–2887 (1980).

Humic soil and coal structure study with magic-angle spinning ^{13}C CP-NMR

P. F. Barron

CSIRO Fuel Geoscience Unit, North Ryde, New South Wales 2113, Australia

M. A. Wilson

Fuel and Atmospheric Chemistry Section, CSIRO Division of Process Technology, North Ryde, New South Wales 2113, Australia

In our recent report on using the ^{13}C cross-polarization (CP) NMR technique to characterize the organic matter in whole soils¹, we discussed the limitations of the technique in terms of resolution and the improvements possible using 'magic-angle' sample spinning (MAS). We now report experiments on a soil and a brown coal in which these techniques have been combined. The spectra provide information on the chemical structure of the materials and throw new light on the nature of the various types of carbon in coals and humic materials.

Although carbonaceous substances such as coals and humic materials are among the most widely distributed and important natural products on the Earth, there have been few techniques available by which their intimate chemical structure could be obtained. However, NMR techniques have allowed semi-quantitative estimations of aromatic carbon in coals¹⁻³ and whole soils¹ to be made. Recently, high power cross-polarization techniques coupled with magic-angle spinning⁴ have improved quantification, through increased resolution, enabling better estimates of the aromaticity of coals⁵ and humic substances⁶. High field techniques have also been used to obtain structural information on coals⁷ and soils⁸, but research here has had to be confined to soluble material.

We have combined the ^{13}C CP-MAS technique with the use of high fields to obtain spectra at 75 MHz for a New Zealand silt loam and an Australian brown coal xylite fraction. The spectra (Fig. 1) consist of many broad resonances, each of which gives information on the structural environments of the carbon atoms present.

A major problem with obtaining ^{13}C CP-MAS spectra is the need to spin at speeds sufficient to remove both the chemical shift anisotropy (CSA) of highly anisotropic carbons (that is, aromatic and carboxyl carbons) and the spinning sidebands from the chemical shift range. As the CSA is field dependent, the required spinning speed increases with increasing field strength and speeds of the order of the CSA are usually considered necessary. At 75 MHz, experiments would have to be performed at speeds in excess of 7 kHz, which are not technologically feasible at present. However, Stejskal *et al.*⁹ have shown that an anisotropy of ~3 kHz is effectively removed at spinning speeds of ~1 kHz but at the expense of an increase in spinning sideband intensities. The spinning speeds we have used at 75 MHz (3.2–3.6 kHz) should be sufficient to remove large anisotropies effectively and hence reveal the expected resolution advantage of high fields. The increased problem of spinning sidebands is difficult to solve but, because their position depends on the spinning rate, their contribution can be reduced by multiplication of spectra obtained at significantly different spinning speeds.

Figure 1a shows the ^{13}C CP-MAS spectrum of Maungatua silt loam. We have previously obtained a 22.6 MHz ^{13}C CP spectrum¹ which indicated that the silt loam was largely aliphatic and highly oxygenated. The high field CP-MAS (3.2 kHz) spectrum confirms these conclusions and provides greater detail.

However, as a consequence of the low carbon content of the soil (22% C) the spectrum is complicated by resonances from the rotor (perdeuterated polymethylmethacrylate⁹).

^{13}C resonances in the spectrum can be assigned if it is assumed that the isotropic chemical shifts (δ) in such solids are not significantly (± 3 p.p.m.) different from those found in solution^{10,11}. The spectrum can be divided into four regions: δ 10–50 p.p.m. (aliphatic carbon), δ 50–110 p.p.m. (oxygenated alkyl carbon), δ 100–160 p.p.m. (aromatic and alkenic carbon) and δ 160–220 p.p.m. (carbonyl carbon).

The strong group of resonances at ~ δ 176 p.p.m. arises from carboxyl carbon in the soil and/or the rotor. Clearly carboxyl carbon is only a minor contributor to this soil. The O-alkyl carbon exhibits signal over the range δ 50–110 p.p.m. with the resolved resonance at δ 74 p.p.m. indicating oxygen-bonded carbons in ethers other than methoxy or ethoxy, or possibly ring carbons in polysaccharides. That the O-alkyl region extends to greater than 100 p.p.m. would be consistent with the presence of polysaccharides¹². Unfortunately, the methoxy and methyl ester region is complicated by strong rotor resonances (δ 55–51 p.p.m.) and therefore we cannot comment on the presence of these groups.

The alkyl region contains clearly defined resonances at δ 29–32 and 15 p.p.m. The range of signals δ 29–32 p.p.m. presumably arises from methylene carbon β , γ , δ and ϵ from terminal methyl groups. The signal at δ 15 p.p.m. arises from terminal methyl carbon of alkyl chains. A small but probable contribution from rotor signals to this resonance, means the only

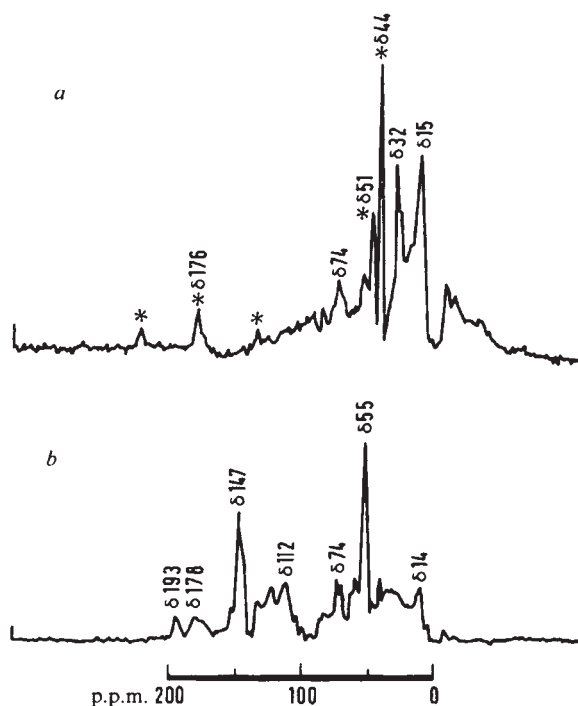


Fig. 1 75 MHz ^{13}C CP-MAS spectra obtained using H_1 fields of 19 and 76 G for ^1H and ^{13}C respectively, with a single contact of 1 ms, on a Bruker CXP-300 spectrometer and Z32 PE/MAS probe. The rotor was machined from perdeuterated polymethylmethacrylate. *a*, Maungatua silt loam. Obtained without block multiplication and using a spinning speed of 3.2 kHz. * Indicates rotor signals and sidebands. *b*, Yallourn brown coal xylite. Obtained by block multiplication of spectra using spinning speeds of 3.2 and 3.6 kHz to reduce spinning side bands. The resulting free induction decay was gaussian resolution enhanced. These materials have been described in detail elsewhere¹. No special procedures were needed for sample preparation other than crushing. Assignments were made by comparison with known compounds^{24,25}. Chemical shifts are relative to external tetramethylsilane and were initially determined relative to external adamantane and are considered accurate to ± 3 p.p.m.

comment we can make on alkyl chain length is that polymethylene is an important constituent of this soil. Our results clearly show, however, the potential of MAS in soil research. We are investigating the potential of other rotor materials for obtaining spectra of whole soils.

Numerous attempts have been made to devise structural formulae which represent the alkali soluble fractions of soil organic matter. These models¹³⁻¹⁵ differ considerably in structure but all involve the idea that soil humic substances are highly aromatic materials.

It has recently been suggested that soil humic substances may not be as aromatic as previously thought. Anderson and Russell¹⁶ suggested that polymaleic-acid-like polymers are important contributors to humic substances. Grant¹⁷ has shown that polymethylene may constitute as much as 30% by weight of humic structures. Signals from aromatic carbons contribute only moderately to the ¹³C NMR spectra of some soil humic materials in solution¹⁸⁻²⁰. Although it may be argued¹⁸ that nuclear Overhauser and relaxation effects prevent quantitative determination from ¹³C NMR of the extract aromaticity, gated ¹H decoupling and significant pulse delays have little effect²⁰ on relative signal intensities of soil humics recorded so far. Our work on whole soils¹ has shown that the soil organic matter present can vary from highly aliphatic to at least moderately aromatic. The soil investigated in the present study is clearly highly aliphatic. Hence the structure models proposed by Fuchs¹³, Felbeck¹⁴, Dragunov¹³ and Schnitzer¹⁵ are not applicable to this soil, though they may adequately represent the organic matter in extracts from other soils.

Figure 1b is the spectrum obtained from a Yallourn brown coal xylite. Previous examination of this material¹ by the ¹³C CP technique (without MAS) at the relatively low frequency of 22.6 MHz showed that the xylite contained considerable amounts of aromatic, O-alkyl and alkyl carbon. The presence of highly anisotropic aromatic carbon would be expected to lead to significant sideband problems. Hence, Fig. 1b is the result of the multiplication of spectra obtained at two spinning speeds to reduce this problem. The 75 MHz ¹³C CP-MAS spectrum exhibits considerable detail and confirms the previous conclusions.

The broad strong resonances present in the spectrum are clearly not from spinning side bands. The resonance at $\delta 147$ arises from aromatic carbons bound to oxygen whereas that at $\delta 112$ is indicative of carbon *ortho* to C_{Ar}-O or of alkenic carbons. The major resonance at $\delta 123$ p.p.m. is due to C_{Ar}-H carbons.

The region $\delta 50$ – 100 p.p.m. can be assigned to the O-alkyl carbon of alcohols, ethers and esters. Resonance from $\delta 70$ – 90 p.p.m. can in general be assigned to carbon α to oxygen where the alkyl group contains more than two carbons. The spectrum exhibits signals over this whole range. Primary alcohols, ethoxy ethers and ethyl esters resonate over the range $\delta 60$ – 65 p.p.m. and small amounts of these materials may be present. The strong resonance at $\delta 55$ p.p.m. can be assigned to methoxy carbons. The presence of large amounts of methoxy groups has been confirmed by pyrolysis-gas chromatography and mass spectroscopy.

The alkyl region extending from $\delta 10$ to $\delta 50$ p.p.m. is not dominated by any alkyl types. There is no large polymethylene peak at $\delta 29$ p.p.m. Zilm *et al.*⁵ obtained the 15.1 MHz ¹³C CP-MAS spectrum of an American lignite and identified a major peak at $\delta 29$ p.p.m. which is readily assigned to polymethylene (CH₂)_n. There is only a very minor peak at $\delta 29$ p.p.m. in the xylite spectrum. It follows that the alkyl chains in Yallourn brown coal xylite are short and branched whereas the alkyl chains in the American coal are much longer. Hence our results indicate the variability of alkyl structures in brown coal components.

Assigning resonances to carboxyl and carboxylic carbon is complicated by the uncertainty of the contribution of spinning side bands to the spectrum but brown coals are known to contain carboxylic carbon. Although the peak at 165 – 180 p.p.m. could

be assigned to carboxylic carbon it may also arise from a residual spinning side band signal. The peak at $\delta 193$ p.p.m. possibly represents ketone carbon, although residual spinning side bands may contribute to this region. Ketone and carboxylic carbon were, however, also identified in pyrograms.

It is generally believed^{21,22} that brown coals contain considerable amounts of carboxylic acid, ether and phenolic functional groups bound to aromatic structures of one or two rings. Our results show the important contribution of methoxy ethers in brown coal xylite. The very strong resonance at $\delta 55$ p.p.m. highlights the importance of methoxy ethers in this brown coal. The unequivocal assignments made here (to those signals which cannot arise from spinning side bands) are also those found in lignin²³. This strongly suggests that Yallourn xylite is a modified lignin.

We are not aware of any reported NMR spectra of whole soils or solid coal which permit the detailed structural assignments achieved here. The present results show the potential and problems of high field high power NMR techniques in soil and coal chemistry.

We thank Drs D. Muller and H. Forster, and Bruker Analytische Messtechnik GMBH, Karlsruhe, FRG for obtaining the CP-MAS spectra. P.F.B. was supported under the National Energy Research, Development and Demonstration program administered by the Commonwealth Department of National Development.

Received 7 July; accepted 3 November 1980.

1. Barron, P. F., Wilson, M. A., Stephens, J. F., Cornell, B. A. & Tate, K. R. *Nature* **286**, 585–587 (1980).
2. Retcofsky, H. L. & VanderHart, D. L. *Preprints 1976 Coal Chemistry Workshop*, 202–217 (Stanford Research Institute, Menlo Park, 1976).
3. Vanderhart, D. L. & Retcofsky, H. L. *Fuel* **55**, 202–204 (1976).
4. Schaefer, J. & Stejskal, E. O. *J. Am. chem. Soc.* **98**, 1031–1032 (1976).
5. Zilm, K. W., Pugmire, R. J., Grant, D. M., Wood, R. E. & Wiser, W. H. *Fuel* **58**, 11–16 (1979).
6. Hatcher, P. G., VanderHart, D. L. & Earl, W. L. *Org. Geochem.* **2**, 87–92 (1980).
7. Bartle, K. D. *Rev. Pure appl. Chem.* **22**, 79–113 (1972).
8. Wilson, M. A., Jones, A. & Williamson, B. F. *Nature* **276**, 487–489 (1978).
9. Stejskal, E. O., Schaefer, J. & McKay, R. A. *J. Magn. Reson.* **25**, 569–573 (1977).
10. Lippmaa, E. T., Alla, M. A., Pehk, T. J. & Engelhardt, G. *J. Am. chem. Soc.* **100**, 1929–1931 (1978).
11. Earl, W. L. & VanderHart, D. L. *Macromolecules* **12**, 762–767 (1978).
12. Voelter, W. & Breitmaier, E. *Org. magn. Reson.* **5**, 311–319 (1973).
13. Kononova, M. M. *Soil Organic Matter* 2nd edn, 73–75 (Pergamon, New York, 1976).
14. Felbeck, G. T. Jr *Adv. Agron.* **17**, 327–333 (1965).
15. Schnitzer, M. & Khan, S. Y. *Soil Organic Matter*, 45–47 (Elsevier, New York, 1978).
16. Anderson, H. A. & Russell, J. D. *Nature* **260**, 597 (1976).
17. Grant, D. *Nature* **270**, 709–710 (1977).
18. Wilson, M. A. & Goh, K. M. *J. Soil Sci.* **28**, 645–652 (1977).
19. Ogner, G. *Soil Biol. Biochem.* **11**, 105–108 (1979).
20. Newman, R. H., Tate, K. R., Barron, P. F. & Wilson, M. A. *J. Soil Sci.* (in the press).
21. Wender, I. *Catal. Rev. Sci. Engng* **14**, 97–129 (1976).
22. Given, P. H. *Fuel* **39**, 147–153 (1960).
23. Mikinis, F. P., Maciel, G. E. & Bartuska, V. J. *Org. Geochem.* **1**, 169–176 (1979).
24. Levy, G. C. & Nelson, G. L. *Carbon-13 Nuclear Magnetic Resonances for Organic Chemists* (Wiley-Interscience, New York, 1976).
25. Stothers, J. B. *Carbon-13 NMR Spectroscopy* (Academic, New York, 1976).

Was the Laramide orogeny related to subduction of an oceanic plateau?

Richard F. Livaccari, Kevin Burke & A. M. C. Şengör

Department of Geological Sciences, State University of New York at Albany, Albany, New York 12222

Numerous models have been presented to explain the late Cretaceous/early Cenozoic Laramide orogeny, which affected the foreland region of the western cordillera within the US¹. The most attractive models invoke low-angle subduction²⁻⁴, which can develop for various reasons^{5,6}. Evidence from South America indicates that one such reason may be the subduction of buoyant ocean floor. We here extend the low-angle subduction models by attributing the shallowing of the subduction angle during the Laramide orogeny to subduction of a large oceanic plateau of anomalously thick and buoyant oceanic crust. Pacific plate history indicates that a twin to the Hess Rise may represent the oceanic plateau that triggered Laramide events.

Large regions of anomalously shallow ocean floor, 100,000 km² or more in area, are found in the Pacific, Indian and Caribbean ocean basins. These regions were formed between the late Jurassic and the late Cretaceous and lie at depths several kilometres shallower than contemporaneous normal oceanic crust⁷. The plateaus have been interpreted as composed of abnormally thick and more buoyant oceanic crust⁸.

Regions such as the Ontong Java and Manihiki plateaus, the Hess Rise and the eastern portion of mid-Pacific mountains seem to have formed by areally extensive, on ridge-axis, volcanic events between 120 and 90 Myr BP (Barremian to Turonian)⁹⁻¹¹. Conversely, the western portion of the mid-Pacific mountains and the present oceanic crust of the Caribbean area seem to be a result of thickening of normal oceanic crust by voluminous off ridge-axis volcanic activity⁹⁻¹¹. The shallowness of the Caribbean ocean floor is related to the late Cretaceous intrusion of large volumes of sills that thickened and made more buoyant the oceanic crust of this area⁸. Plateau regions that are generated by intense on ridge-axis volcanism may form symmetrical objects or 'twins' on two plates¹².

Regions of unusually buoyant ocean floor are difficult to subduct^{8,13-15}. Therefore, the subduction or attempted subduction of oceanic plateaus or linear aseismic ridges can be expected to have a profound effect on the internal tectonics of the associated arc^{8,13-15}.

Because oceanic plateau regions are composed of crust that has a buoyancy between normal oceanic crust and continental crust, the subduction of such plateaus will cause the subducted lithospheric plate to ride higher in the asthenosphere, considerably lowering the angle of subduction¹⁵. For example, beneath the South American Cordillera the subducted oceanic plate has been segmented along strike, resulting in alternating sections of high- and low-angle subduction of the same plate¹⁶. Segments characterized by low-angle subduction correspond with portions of the cordilleran belt that lack arc-related Quaternary volcanism. Above the segments of high-angle subduction, Quaternary volcanism is abundant. An extremely important observation is that the segments of low-angle subduction beneath Peru and central Chile correspond with places where the eastern extensions of the Nazca and Juan Fernandez aseismic ridges have been subducted¹⁶⁻¹⁹. This suggests that the thickened and more buoyant oceanic crust of both the Nazca and Juan Fernandez aseismic ridges causes the descending oceanic plate to ride higher in the asthenosphere, resulting in a lower angle of

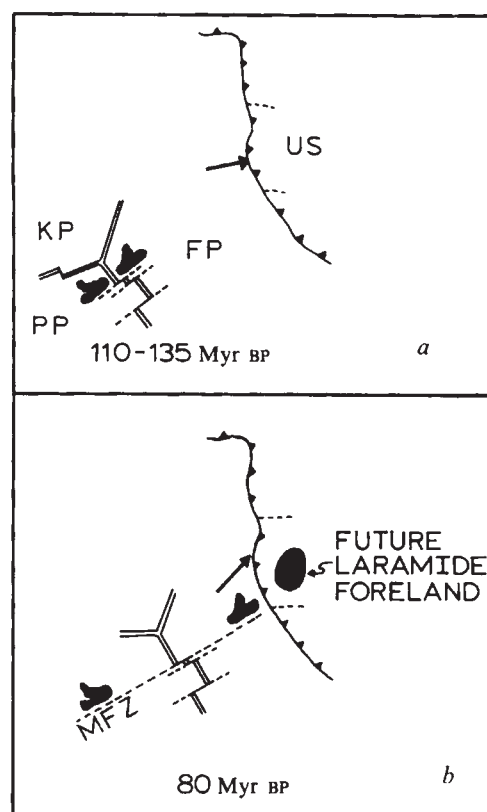


Fig. 2 Cretaceous reconstructions of the Kula, Farallon, Pacific and North American plates (modified from ref. 33): *a*, The formation of the Hess Rise and Hess Rise 'twin' by an unusually intense on axis volcanic event during the early Cretaceous; *b*, the movement of the Hess Rise 'twin' towards the future Laramide foreland during medial and late Cretaceous. Black arrows show the relative motion between the North American and Farallon plates³³ (KP, Kula Plate; FP, Farallon Plate; PP, Pacific Plate; MFZ, Mendicino Fracture Zone).

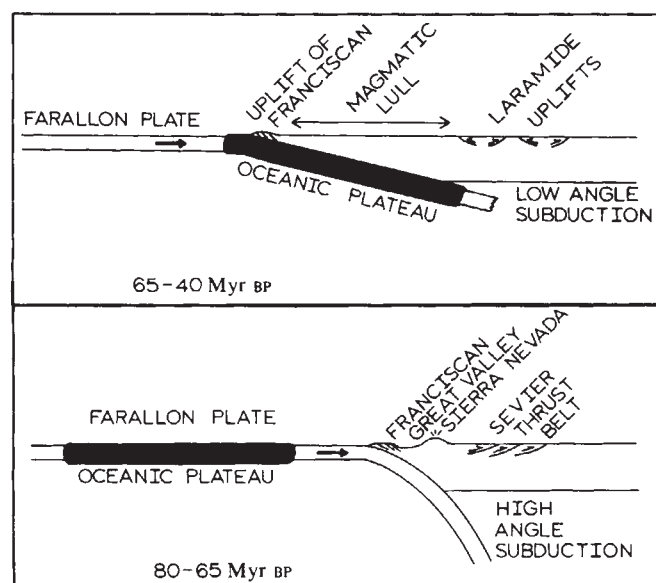


Fig. 1 Schematic cross-sections of the western US cordillera illustrating the progressive approach of an oceanic plateau towards the western US during the medial Cretaceous times and eventual subduction of the plateau during the Laramide orogeny.

subduction^{17,19}. Enhanced seismic activity in the upper 50 km of the overriding South American plate²⁰ and extensive high- and low-angle thrust faulting in the foreland regions of Peru and Ecuador up to 700 km east of the South American trench^{19,21,22} are associated with these sections of low-angle subduction.

The Laramide orogeny was characterized by the development of basement-cored overthrusts and adjacent synkinematic depositional basins in the foreland of the western US between northern Montana and northern New Mexico²³. Deformation began ~70 Myr BP and continued until ~40 Myr BP (refs 24, 25). To the north and south of the Laramide foreland, Sevier-type retroarc thrusting continued^{4,25}. The Laramide orogeny was a non-collisional, compressional event that resulted from the collapse and telescoping of the entire foreland region between Montana and New Mexico²⁶. Faults that bound the ranges seem, mostly, to be low-angle thrusts that do not steepen at depth, indicating that horizontal compression and collapse of the foreland region was responsible for development of Laramide structures^{22,27}. To the west of the Laramide foreland, arc volcanism ceased along the Sierra Nevada²⁸ and migrated inland, implying a rapid shallowing in the angle of subduction of the Farallon plate beneath the western US^{29,30}.

All these events may be interpreted as being the result of subduction of an oceanic plateau adjacent to this region. Subduction of a plateau of approximately the same dimensions as the Laramide foreland would result in flattening of the subduction angle, cessation of arc volcanism and increased compressive deformation, resulting in collapse in the foreland region (Figs 1 and 2). Therefore, tectonic patterns that characterized the Laramide orogeny such as cessation of arc volcanism and intense foreland deformation may be explained by inferring

the subduction of an oceanic plateau adjacent to the Laramide foreland in the late Cretaceous/early Palaeogene, analogous with the situation presently found in South America.

Although it cannot be directly proved that such an oceanic plateau region ever existed or was subducted beneath the western US, the formation of numerous oceanic plateau regions in Cretaceous times, such as the Ontong Java and Manihiki plateaus, the mid-Pacific mountains and the present oceanic crust of the Caribbean, implies that these and perhaps other plateau regions lay within the Pacific Ocean during the late Mesozoic.

The Hess Rise is an oceanic plateau up to 800 km across lying just north of the westernmost extension of the Mendocino fracture zone and just south of the Great Magnetic Bight³¹ (Fig. 2). The oldest sediments found on the Hess Rise are early Albian in age^{10,32}, implying that the Rise was formed sometime in the early Cretaceous. Watts *et al.*¹¹ and Vallier *et al.*¹⁰ have presented evidence demonstrating that the Hess Rise formed by an intense on-axis volcanic event between 120 and 90 Myr BP (Barremian to Turonian). If this was the case, then symmetrical plateau twins may have been generated on two plates¹² (Fig. 2). That is, a Hess Rise twin could have formed on the Farallon plate symmetrically with the Hess Rise on the Pacific Plate.

Figure 2 shows reconstructed plate geometries for the Cretaceous³³ and the approximate positions of both the Hess Rise and postulated Hess Rise twin. Symmetrical spreading about the Pacific–Farallon ridge brings the Hess Rise twin into a position just opposite the future Laramide foreland by the late Cretaceous (Fig. 2). Continued convergence between the North American and Farallon plates at the rapid rate of $\sim 14 \text{ cm yr}^{-1}$ (ref. 33) would have resulted in subduction of a Hess Rise twin opposite the Laramide foreland during the late Cretaceous through the early Cenozoic. Thus, subduction of a Hess twin beneath the western US may have been the cause of the distinctive Laramide orogeny.

Indirect evidence within the Cordillera for the subduction of an oceanic plateau beneath the western US during the Laramide orogeny may exist. Because plateau regions lie at depths 2–3 km above oceanic crust of the same age, then during the subduction of an oceanic plateau, uplift would be expected along the entire fore-arc region as it rose to accommodate oceanic crust riding at a much higher elevation. Dickinson *et al.*³⁴ have documented such an event for the forearc region of California in the late Cretaceous that lifted the trench slope-break to a position near sea level resulting in widespread subaerial unconformities and filling of the fore-arc trough to the east to form a wide fore-arc platform area. Also, Winterer³⁵ has suggested that the Calera and Laytonville limestones found within the Franciscan melange may represent obducted slices of an oceanic plateau.

We thank J. F. Dewey, R. H. Pilger and W. R. Dickinson for preprints, and R. V. Ingersoll, R. H. Pilger, T. A. Cross and W. S. F. Kidd for reviews. Research at the State University of New York at Albany on oceanic plateaus has been partly supported by NSF grants 7614754 and 7803319.

Received 30 May; accepted 11 November 1980.

- Woodward, L. A. *New Mex. geol. Soc. Spec. Publ.* **6**, 11 (1976).
- Coney, P. J. *Am. J. Sci.* **272**, 603 (1972).
- Dickinson, W. R. & Snyder, W. S. *Geol. Soc. Am. Mem.* **151**, 355 (1978).
- Burchfiel, B. C. & Davis, G. A. *Am. J. Sci.* **275A**, 363 (1975).
- Molnar, P. & Atwater, T. *Earth. planet. Sci. Lett.* **41**, 330 (1978).
- Dewey, J. F. *Geol. Soc. Can. Spec. Publ.* **20**, (in the press).
- Sclater, J. G., Anderson, R. N. & Bell, M. L. *J. geophys. Res.* **76**, 7888, (1971).
- Burke, K., Fox, P. J. & Sengör, A. M. C. *J. geophys. Res.* **83**, 3949 (1978).
- Winterer, E. L. *Am. geophys. Un. Monogr.* No. 19, 269 (1976).
- Vallier, T. L. *et al. Nature* **286**, 48 (1980).
- Watts, A. B., Bodine, J. H. & Ribe, N. M. *Nature* **283**, 532 (1980).
- Sharmar, G. F. *EOS* **60**, 949 (1979).
- Vogt, P. R., Lowrie, A., Bracey, D. R. & Hey, R. N. *Geol. Soc. Am. Spec. Pap.* 172 (1976).
- Hughes, G. W. & Turner, C. C. *Bull. geol. Soc. Am.* **88**, 412 (1977).
- Kelleher, J. & McCann, W. L. *Am. geophys. Un. Maurice Ewing Ser.* **1**, 115 (1977).
- Isacks, B. L. & Barazangi, M. *Am. geophys. Un. Maurice Ewing Ser.* **1**, 99 (1977).
- Pilger, R. H. *EOS* **58**, 1232 (1977).
- Aleman Ramirez, A. M. & Pilger, R. H. *EOS* **59**, 383 (1978).
- Pilger, R. H. *Bull. geol. Soc. Am.* (in the press).
- Barazangi, M. & Isacks, B. L. *Geology* **4**, 686 (1976).
- Burchfiel, B. C. & Davis, G. A. *Nature* **260**, 693 (1976).
- Brewer, J. A., Smithson, S. B., Oliver, J. E., Kaufman, S. & Brown, L. D. *Tectonophysics* **62**, 165 (1980).

- Hamilton, W. *Soc. Econ. Paleont. Miner. Pacific Coast Paleogeogr. Symp.* **2**, 33 (1978).
- Tweto, O. *Geol. Soc. Am. Mem.* **144**, 1 (1975).
- Armstrong, R. L. *Nature* **247**, 348 (1974).
- Coney, P. J. *New Mex. geol. Soc. Spec. Publ.* **6**, 5 (1976).
- Smithson, S. B., Brewer, J., Kaufman, S., Oliver, J. & Hurich, C. *Geology* **6**, 648 (1978).
- Snyder, W. S., Dickinson, W. R. & Silberman, M. L. *Earth planet. Sci. Lett.* **32**, 91 (1976).
- Coney, P. J. & Reynolds, S. J. *Nature* **270**, 403 (1977).
- Cross, T. A. & Pilger, R. H. Jr *Am. J. Sci.* **278**, 865 (1978).
- Chase, T. E., Menard, H. W. & Mammerickx, J. *I.M.R. Tech. Rep. Ser.* TR-17 (1971).
- Scientific Party Init. *Rep. DSDP Leg 32*, 233 (1975).
- Coney, P. J. *Geol. Soc. Am. Mem.* **152**, 33 (1978).
- Dickinson, W. R., Ingersoll, R. V. & Graham, S. A. *Bull. geol. Soc. Am.* **90**, 1458 (1979).
- Winterer, E. L. *Texas A & M Univ. Symp. on Oceanic Plateaus*, 15 (1979).

Old model Nd ages in Namibian Pan-African rocks

C. J. Hawkesworth*, J. D. Kramers† & R. McG. Miller‡

* Department of Earth Sciences, The Open University, Walton Hall, Milton Keynes MK7 6AA, UK

† Department of Earth Sciences, The University, Leeds LS2 9JT, UK

‡ Geological Survey, PO Box 2168, Windhoek, 9100, Namibia

The Nd-isotope analytical technique is a powerful tool for studying many geological processes: particularly the evolution of the upper mantle as seen through the isotope and trace element geochemistry of mantle-derived volcanic rocks^{1–3}, and magmatic processes along destructive plate margins^{4,5}. However, previous studies of continental crust^{6–8} have been largely restricted to Archaean examples and concerned primarily with dating their time of derivation from the upper mantle. In the present study we investigated the Nd- and Sr-isotope characteristics of rocks involved in a relatively young (800–450 Myr) orogenic event. Such events represent critical stages in the evolution of most of the Earth's continental crust—new material is added from the mantle, and pre-existing crust is remobilized by erosion and sedimentation, deformation and magmatic activity. Nd- and Sr-isotopes were used to outline the range in age and geochemical characteristics of rock sequences and provinces within both the upper mantle and the pre-existing crust which were sampled during the orogeny, and hence provided the major components of what is now a stable segment of continental crust.

The Damara high-temperature belt of Namibia (Fig. 1a), formed during the widespread orogenic event first categorized as Pan African by Kennedy⁹ and it was chosen because it is arguably the best exposed orogenic belt of its age and has been studied in detail both geologically and geochemically (refs 10–13 and unpublished results). Some aspects of the stratigraphy and in particular the age and regional significance of the earlier tectonic events are still poorly understood, but an outline is presented in Fig. 1b.

Radiometric ages on pre-Damara rocks range from 1,100 Myr along the southern margin^{14,15} to 1,700–2,000 Myr in the centre of the belt¹⁶ and near the northern margin¹⁷. Within the Damara succession, the first major magmatic event is recorded in a suite of large ionic lithophile (LIL)-element-enriched lavas (Naauwpoort volcanics) and associated syenites and carbonatites exposed along the northern margin. U/Pb zircon results are variable, but they seem to cluster around an age of 800 Myr (ref. 18). In the north, Damara sediments reflect a well developed shelf facies, whereas in the centre and south, clastic sediments with only minor carbonates are followed by a thick flysch-type succession (Kuseb schists). Within the flysch there is a thin band of basic rock, the Matchless amphibolite, which has been interpreted both as a fragment of tectonically emplaced ocean floor material¹⁹, and as just one of several basic volcanic units within the flysch trough¹⁰. Whichever interpretation is correct an Rb/Sr whole-rock age of 765 ± 37 Myr

from this Matchless amphibolite (A. Kröner, personal communication) is probably a minimum age for most of the sediments still preserved in the southern zone.

Granitic rocks, varying from diorites to granites and highly potassic alaskites, dominate the centre of the Damara belt (Fig. 1). Some, which intrude Damara sediments and have undergone extensive regional deformation, yield ages of 700–750 Myr (A. Kröner, personal communication). They are loosely termed 'early' granites and their relationship to later events is not understood. 'Late' granites, by contrast (Fig. 1b), all post-date the major regional deformation and many have been studied previously (refs 12, 20, 21 and unpublished results). Initial Sr-isotope ratios vary from 0.705 to 0.759 (unpublished results) and many of the younger granites, which tend to have the higher initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios were probably emplaced during late uplift in the centre of the belt.

The Damara orogenic cycle may therefore be broadly subdivided into four phases: (1) clastic sedimentation and LILElement-enriched magmatism; (2) basic magmatism (possibly ocean floor) and predominantly flysch-type sedimentation; (3) early granites and regional tectonism; and (4) late, largely post-tectonic granites with associated high-temperature metamorphism. Our samples included volcanic and sedimentary rocks and a range of lithologies from the late granites (Fig. 1).

$^{143}\text{Nd}/^{144}\text{Nd}$ ratios and the concentration of eight rare earth elements (REEs) were determined using techniques described previously⁴ and the results are presented in Tables 1 and 2. Except for K211, all samples were selected from suites which plot on whole-rock Rb–Sr isochrons (unpublished results). Their initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are thus representative of the particular rock unit. Initial Nd-isotope ratios vary from 0.51196 in the Matchless amphibolite to 0.51117 in the K_2O -rich Rössing alaskite. Figure 2 shows chondrite-normalized REE patterns for selected samples.

The carbonatite (28/32) from the Naauppoort magmatic suite contains extremely high REE concentrations similar to

those reported from carbonatites elsewhere²². Although we are unaware of other carbonatites with a similar 'concave-up' distribution (Fig. 2), such patterns are predicted by experimental work on the distribution of REEs between common mantle minerals and a CO_2 -rich vapour phase²³.

The samples of Matchless amphibolite (AM 62, 63) exhibit higher Sm/Nd and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios than the chondritic uniform reservoir (CHUR², presumed to be representative of the bulk Earth) at the time of their formation. This indicates that their source region had been relatively depleted in light REEs for a considerable time and, because such depletion is characteristic of recent mid-oceanic ridge basalts, these results are in keeping with the interpretation that the Matchless amphibolite represents a fragment of oceanic crust¹⁹. The Gariep Belt is another possible ophiolite sequence, probably of similar age to the Matchless, and whereas the dolerite sample (K211) is enriched in light REEs, it exhibits the same relatively high initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratio (Table 1).

Kh 24 (Kuseb schist) is a metamorphosed flysch-like sediment which was probably deposited 700–800 Myr ago (Fig. 1). Rb/Sr whole-rock results scatter about an 'errorchron' corresponding to an age of 548 ± 56 Myr, which has been interpreted as the time of widespread regional metamorphism (unpublished results). The REEs exhibit a negative Eu anomaly which is typical of post-Archaeon sediments²⁴ and a Sm/Nd ratio in the range suggested for average continental crust²⁵ (0.17–0.21).

The Damara 'granites' sampled are from massive stocks of diorite and granite and from small bodies of K-rich alaskite. The diorite (RM586) and two granites (RM662 and SM2) range in age from 560 to 490 Myr, in initial $^{87}\text{Sr}/^{86}\text{Sr}$ from 0.7048 to 0.709, and in initial $^{143}\text{Nd}/^{144}\text{Nd}$ from 0.51186 to 0.51146. The diorite, with its initial Sr- and Nd-isotope ratios similar to CHUR 550 Myr ago, probably originated in the upper mantle although its generation must have involved considerable fractionation of the light REEs (Fig. 2). The granites have higher

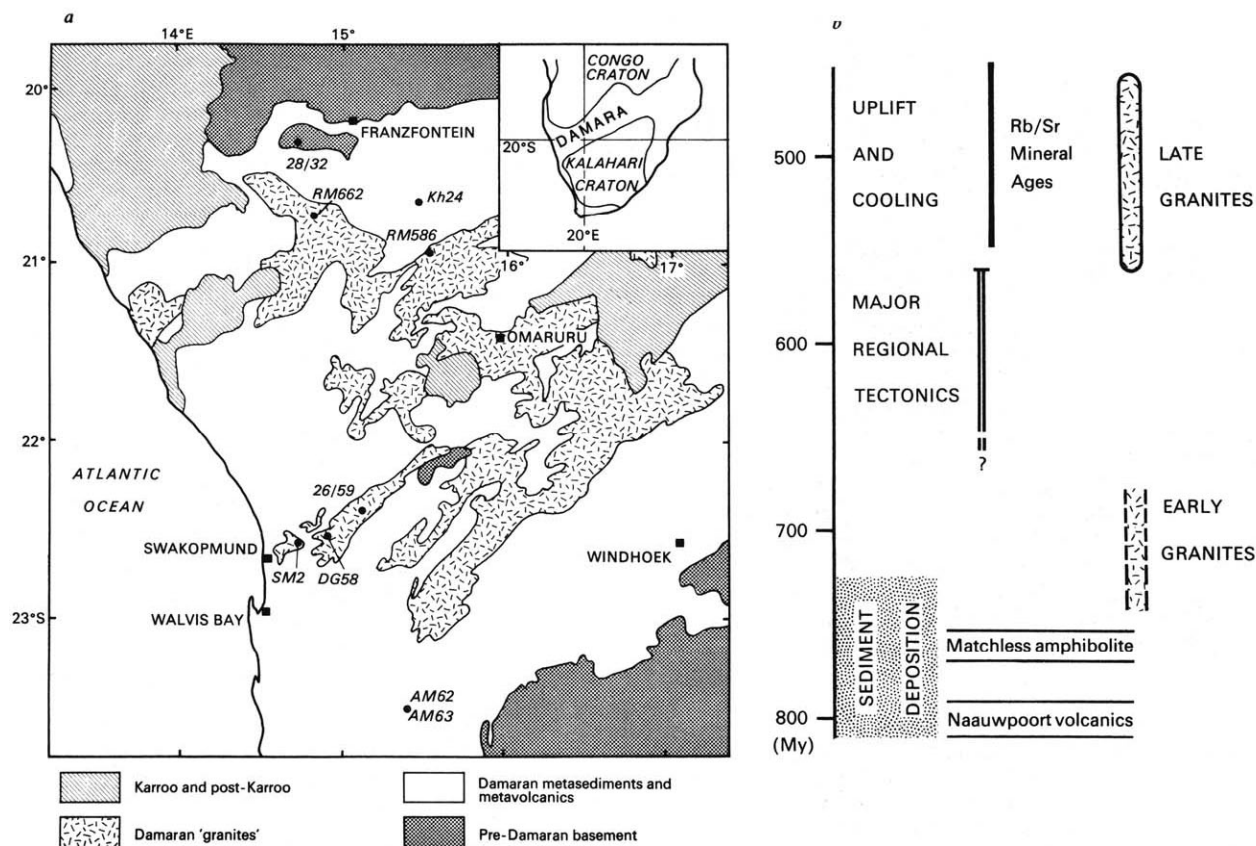


Fig. 1 a, Geological map of the Damara high-temperature belt, Namibia, illustrating sample localities. b, Summary of the major geological events recognized within the central Damara succession.

Table 1 Nd-isotope results

Rock no.	Lithology	a	b	c	d	e	f
		Age (Myr)	$^{143}\text{Nd}/^{144}\text{Nd}_m$	Sm/Nd	$^{143}\text{Nd}/^{144}\text{Nd}_0$	$T_{\text{CHUR}}^{\text{Nd}}$ (Ga)	$^{87}\text{Sr}/^{86}\text{Sr}_0$
AM62	Matchless amphibolite	765 ± 37	0.513124 ± 34	0.366	0.51196	2.5	0.70566 ± 7
AM63	Matchless amphibolite	765 ± 37	0.513093 ± 18	0.365	0.51194	2.4	0.70566 ± 7
K211	Gariiep dolerite	~765	0.512440 ± 26	0.143	0.51196	0.3	—
28/32	Lofdal carbonatite	~800	0.512201 ± 20	0.181	0.51157	0.8	0.7032 ± 3
Kh24	Kuiseb schist	548 ± 56	0.512219 ± 20	0.209	0.51171	1.0	0.715 ± 2
RM586	Salem diorite	~550	0.512330 ± 30	0.178	0.51186	0.6	0.7048 ± 6
SM2	Salem granite	563 ± 63	0.512153 ± 28	0.189	0.51168	1.0	0.707 ± 4
RM662	Sorris Sorris Granite	495 ± 15	0.511795 ± 16	0.144	0.51146	1.2	0.709 ± 1
26/59–155.5m	Valencia alaskite	484 ± 18	0.511735 ± 24	0.219	0.51126	2.3	0.724 ± 2
DG58	Rössing alaskite	458 ± 8	0.511588 ± 28	0.204	0.51117	2.3	0.759 ± 1

a, Rb/Sr whole rock ages (unpublished results and A. Kröner, personal communication), except for the Lofdal carbonatite (U/Pb zircon¹⁸) and Gariiep dolerite (assumed to be of the same age as the Matchless amphibolite).

b, Measured $^{143}\text{Nd}/^{144}\text{Nd}$ ratios. Normalized to $^{146}\text{Nd}/^{144}\text{Nd} = 0.7219$. Quoted errors are two standard errors on the mean. $^{143}\text{Nd}/^{144}\text{Nd}$, BCR-1 = 0.51266 ± 2.

c, From Table 2, except AM63 (R.M., unpublished results).

d, Initial $^{143}\text{Nd}/^{144}\text{Nd}$ normalized to BCR-1 = 0.51262.

$$e, T_{\text{CHUR}}^{\text{Nd}} = \frac{1}{\lambda} \ln \left[\frac{^{143}\text{Nd}/^{144}\text{Nd}_{\text{CHUR}} - ^{143}\text{Nd}/^{144}\text{Nd}_{\text{sample}}}{^{147}\text{Sm}/^{144}\text{Nd}_{\text{CHUR}} - ^{147}\text{Sm}/^{144}\text{Nd}_{\text{sample}}} + 1 \right]$$

where $\lambda = 6.54 \times 10^{-12} \text{ yr}^{-1}$, $^{143}\text{Nd}/^{144}\text{Nd}_{\text{CHUR}} = 0.51262$, $^{147}\text{Sm}/^{144}\text{Nd}_{\text{CHUR}} = 0.1935$. The $T_{\text{CHUR}}^{\text{Nd}}$ age for K211 is meaningless as its Sm/Nd ratio is probably much less than that in its source rock, whereas those for 26/59 and DG58 are probably slightly too old (see text).

f, Initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (unpublished results and A. Kröner, personal communication).

initial $^{87}\text{Sr}/^{86}\text{Sr}$ and lower initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios and thus probably contain material with a crustal prehistory. Their much larger Eu anomalies (Fig. 2) show that their generation involved either separation from a plagioclase-bearing residue, or plagioclase fractionation. Alaskites in contrast were produced by partial melting at high levels in the crust, sometimes apparently *in situ*²⁶. Samples 26/59 and DG58 are from 480- and 460-Myr old bodies with unusually high initial $^{87}\text{Sr}/^{86}\text{Sr}$ (0.724, 0.759) (ref. 21 and unpublished results) and low initial $^{143}\text{Nd}/^{144}\text{Nd}$ (0.51126, 0.51117) ratios. Note the low concentrations of Sr and the medium- and light-REE, especially in the samples from Rössing (DG58 and 59) and the 'concave-up' distribution patterns (Fig. 2). The low Sr concentrations are consistent with a small degree of partial melting in equilibrium with residual plagioclase. The REE distribution patterns could reflect the presence of minor phases in the residuum, or the scavenging of REE with U in the formation of the adjacent U mineralization. Rössing, for example, is the site of a major uranium deposit and monazite, xenotime and sphene (all minerals with very high crystal-liquid partition coefficients for medium- and light-REE)²⁷ are commonly found in association with the uranium mineralization (refs 4, 28, and Marlow, personal communication).

The initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of these Pan African rocks are plotted against time in Fig. 3, with the evolution of the chondritic reservoir (CHUR²) shown for reference. The possible ocean-floor volcanics (amphibolites and dolerite) and the carbonatite and diorite have initial Nd-isotope ratios respectively higher than and similar to that of CHUR at the time of their formation, whereas the late granites and alaskites have progressively lower initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios. Moreover, as there is a broad inverse correlation between initial Sr- and Nd-isotopes (expressed as ϵ_{Nd} and ϵ_{Sr} respectively²⁹, Fig. 4), the lower initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in the younger rocks is matched by their progressively higher $^{87}\text{Sr}/^{86}\text{Sr}$ ratios.

$T_{\text{CHUR}}^{\text{Nd}}$ ages⁸ are estimates of how long a rock (and its source region) have had different Sm/Nd, and hence $^{143}\text{Nd}/^{144}\text{Nd}$ ratios to CHUR. They denote the time at which the Nd-isotope evolution curve of a system with particular present day $^{143}\text{Nd}/^{144}\text{Nd}$ and Sm/Nd ratios intersects that of CHUR (see Fig. 3). $T_{\text{CHUR}}^{\text{Nd}}$ ages are model ages because it is assumed that a simple two-stage history applies. The first stage is in a (mantle) province with the isotope and trace element characteristics of CHUR, and the second stage reflects the $^{143}\text{Nd}/^{144}\text{Nd}$ and

Sm/Nd ratios in the rocks at present. If such a model is inapplicable as, for example, when light REE-enriched crustal rocks are derived from depleted mantle (higher $^{143}\text{Nd}/^{144}\text{Nd}$ than CHUR), the time of separation from the mantle may be

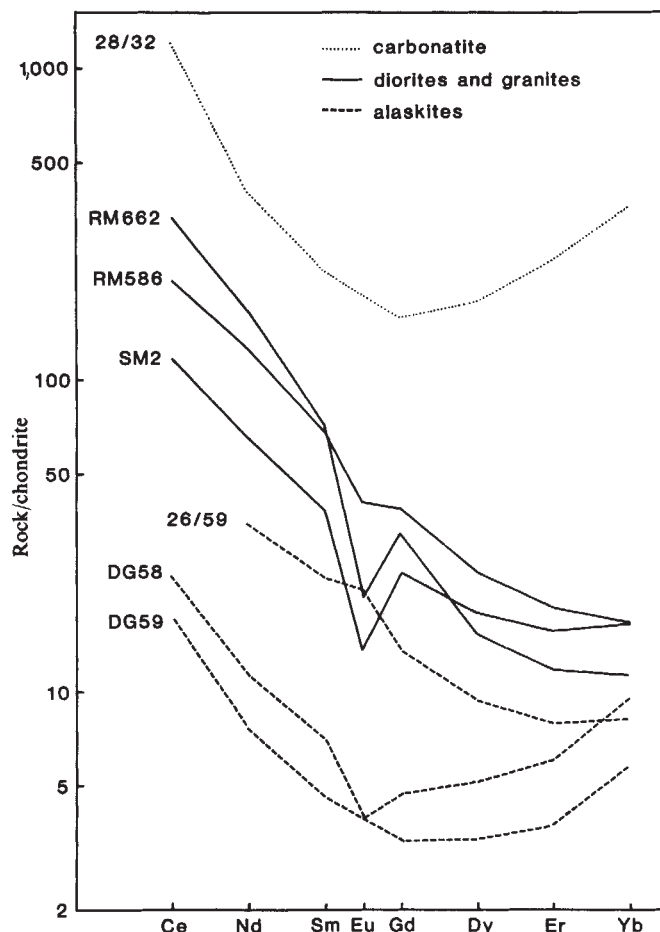


Fig. 2 Chondrite-normalized REE distribution patterns for selected Damara rocks.

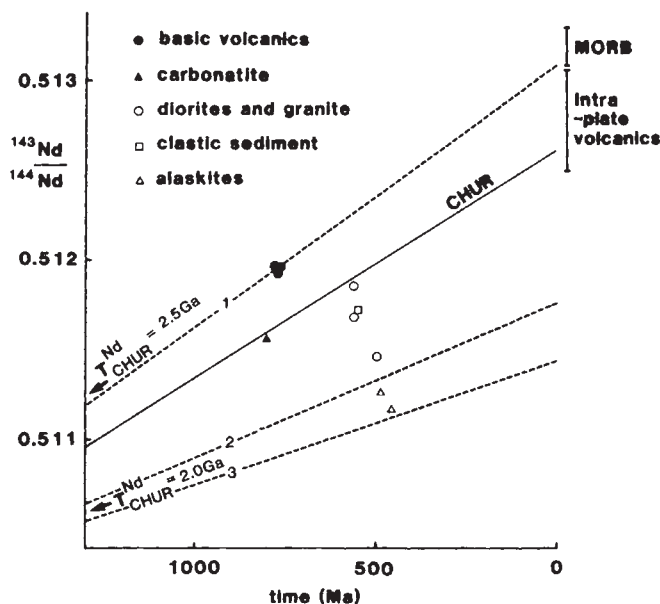


Fig. 3 Variations in initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios in the Damara rocks. CHUR (chondrite uniform reservoir), $^{147}\text{Sm}/^{144}\text{Nd} = 0.1935$, present day $^{143}\text{Nd}/^{144}\text{Nd} = 0.51262$. Evolution paths; 1, $\text{Sm}/\text{Nd} = 0.366$ (as in the samples of Matchless amphibolite); 2 and 3, $\text{Sm}/\text{Nd} = 0.214$ and 0.173 respectively (that is the range in average continental crust²⁵). The samples between evolution paths 2 and CHUR have $T_{\text{CHUR}}^{\text{Nd}}$ ages in the range 550–1,200 Myr (Table 1).

older than that implied by their $T_{\text{CHUR}}^{\text{Nd}}$ age. Nonetheless, for mantle-derived volcanics, such model Nd ages reflect how long chemical variations have persisted in that part of the upper mantle, and for crustal-derived rocks (be they magmatic or sedimentary) they provide a useful estimate of the age of their source region. In all cases they require that the Sm/Nd and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of the rocks are similar to those in their source areas; inevitably this assumption is more valid for some rock types than for others.

The basic volcanic rocks probably reflect 10–20% partial melting of spinel peridotite and thus their Sm/Nd ratios are likely to be similar to those in their source regions³. Thus the evolution path of material with $\text{Sm}/\text{Nd} = 0.366$ (that is, as in AM62) has been plotted in Fig. 3; $T_{\text{CHUR}}^{\text{Nd}}$ equals 2,500 Myr, and its present day $^{143}\text{Nd}/^{144}\text{Nd}$ is similar to those in recent MOR basalts. This suggests that these rocks were derived from mantle which had been depleted in light REE since the Archaean and supports the interpretation that both the Matchless and Gariep Belts represent fragments of oceanic crust¹⁹. The anomalously high ϵ_{Sr} values of these samples (Fig. 4) probably reflects contamination, either with seawater on the ocean floor³⁰, or during post-emplacement metamorphism.

The low concentrations of REEs in the alaskites and 'concave-up' distribution patterns (Fig. 2) indicate that they have probably been modified—perhaps by processes associated

with the uranium mineralization. Thus, rather than assuming that the Sm/Nd ratios of the individual alaskites were the same as their source rocks, we have plotted the evolution of material which was derived from a chondritic reservoir (CHUR) 2,000 Myr ago (that is, the oldest age reported from basement rocks in the Damara¹⁶) and had Sm/Nd ratios in the range for average continental crust (0.173–0.214, see ref. 25 for discussion). Its evolution path in Fig. 3 encompasses the low initial Nd-isotope ratios of the alaskites which strongly suggests that they were derived from crustal material which had been out of the mantle since 2,000 Myr.

The initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios of the granites (SM2, RM662) and the metasediment (Kh24) are intermediate between those of the alaskites and CHUR at the time of their formation (Fig. 3). Apart from equilibrium with minor phases (as presumably happened in the case of the alaskites), equilibration with a large amount of cumulus or residual amphibole is the most likely mechanism capable of altering significantly the Sm/Nd ratios of a crustal melt³¹. This would be reflected in a strong depletion in heavy REEs and, because this is not observed in any of these samples, we conclude that the Sm/Nd ratios of the granites SM2 and RM662 are likely to be similar to those in their source rocks. Sample Kh24, being a metasediment, is also likely to have an Sm/Nd ratio similar to that in its source terrain⁸. The $T_{\text{CHUR}}^{\text{Nd}}$ ages for these two granites and the metasediment range from 1,000 to 1,200 Myr indicating that, unlike the alaskites, they were not derived from 2,000-Myr old crustal material alone. Two possibilities remain: the granites (and the metasediment) could simply have been derived from younger crustal sources (perhaps ~1,100-Myr old) or they could contain variable amounts of both mantle-derived and 2,000-Myr crustal material. For the granites this might reflect contamination of mantle-derived magmas within the crust, whereas the flysch-type sediment could contain a mixture of young volcanic and older continental detritus. At present there are insufficient data to choose between these two hypotheses, however: (1) significant quantities of new crustal material was generated 1,100 Myr ago along the present-day southern margin of the Damara^{14,15}, and crustal rocks of this age may also occur at depth elsewhere within the belt; (2) where contamination of magmas with continental crust has been convincingly demonstrated, it tends to result in a flat-lying trend between Nd- and Sr-isotopes⁵. This is not observed for the Damara diorite and granites (Fig. 4).

The inverse correlation of ϵ_{Sr} and ϵ_{Nd} observed in the crustal rocks studied is interesting. The inverse correlation in mantle-derived volcanics (also shown in Fig. 4) is generally accepted as an expression of coherent fractionation of Rb/Sr and Nd/Sm ratios during melting in the mantle. However, magmatic and sedimentary processes, particularly in the upper crust, fractionate Rb/Sr much more readily than Nd/Sm. Thus sediments and K₂O-rich granitic material develop with time relatively high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, resulting in a distinctive, curved inverse correlation between ϵ_{Nd} and ϵ_{Sr} at higher levels in the continental crust (Fig. 4). Furthermore, although magmatism in this orogenic event has sampled material from the upper mantle through to the upper continental crust, it does not seem to have sampled

Table 2 Trace elements (p.p.m.)

Rock no.	Rb*	Sr*	Rb/Sr	Ce	Nd	Sm	Eu	Gd	Dy	Er	Yb
AM62	—	—	—	8.44	7.85	2.87	1.01	4.16	5.15	3.41	3.17
K211	105	153	0.686	374	88	12.6	2.98	9.69	9.50	5.60	5.61
28/32	9.4	6,660	0.0014	1,062	249	45.1	14.2	43.9	61.7	54.1	77.9
Kh24	78	139	0.561	101	46.9	9.80	1.78	7.95	8.42	5.13	5.21
RM586	71	1,045	0.68	180	79	14.1	3.10	10.9	8.25	4.17	3.70
SM2	183	126	1.45	103	41.2	7.79	1.06	6.72	6.13	3.50	3.60
RM662	213	205	1.04	293	101	14.5	1.59	8.81	5.21	2.65	2.46
26/59	250	123	2.03	—	21.6	4.73	1.65	3.76	3.21	1.78	1.83
DG58	334	28	11.9	20.3	7.04	1.44	0.30	1.28	1.78	1.37	2.11
DG59	315	18	17.5	15.2	4.78	0.94	0.31	0.93	1.16	0.85	1.28

* Unpublished results.

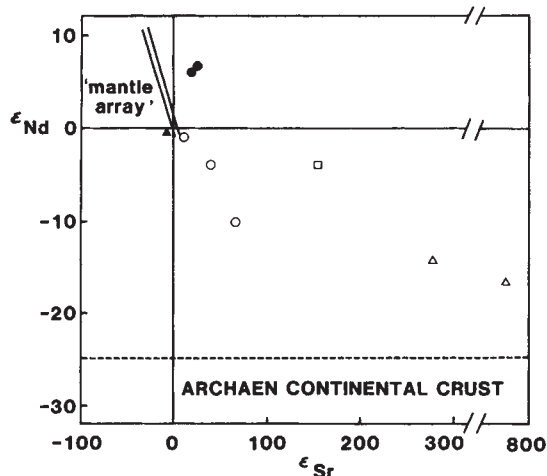


Fig. 4 ϵ_{Nd} against ϵ_{Sr} (ref. 2) for Damara rocks (key as for Fig. 3)

$$\epsilon_{Nd} = \left[\frac{^{143}\text{Nd}/^{144}\text{Nd}(\text{initial})}{^{143}\text{Nd}/^{144}\text{Nd}_{\text{CHUR}(t)}} - 1 \right] \times 10^4$$

where $^{143}\text{Nd}/^{144}\text{Nd}_{\text{CHUR}(t)}$ is the $^{143}\text{Nd}/^{144}\text{Nd}$ ratio of a chondritic uniform reservoir at the time of formation of the rock in question. ϵ_{Sr} is similarly defined using the relevant $^{87}\text{Sr}/^{86}\text{Sr}$ ratios². ϵ_{Nd} and ϵ_{Sr} calculated using the ages and initial Nd- and Sr-isotope ratios in Table 1, but note that the age of the sediment (Kh24) is believed to reflect regional metamorphism (unpublished results).

any old granulite facies rocks ($-\epsilon_{Nd}$ and $-\epsilon_{Sr}$, Fig. 4, refs 1, 7). Such rocks may not be ubiquitous in the lower crust, but it is also possible that such LIL-element depleted material is not readily mobilized.

We conclude that Pan African rocks in Namibia were derived from upper-mantle and continental sources which at the time of the Damara orogeny had already been respectively depleted and enriched in light rare earths for up to 2,000 Myr. High initial $^{143}\text{Nd}/^{144}\text{Nd}$ ratios on basic volcanic rocks support suggestions that they represent fragments of oceanic crust¹⁸, and imply that their source regions had been depleted in light REEs since the Archaean. $T_{\text{CHUR}}^{\text{Nd}}$ ages of the granitic rocks range from 550 to 2,000 Myr. The older ages are from alaskites which, on field evidence, were generated at higher levels in the crust. The lower crust seems to contain younger (?1,100 Myr) crustal material. More generally this evidence for the remobilization of 2,000-Myr old crust highlights an important difference between high-temperature intracratonic Pan-African belts such as the Damara (Fig. 1) and those in north-east Africa which appear to be less deeply eroded, contain no evidence for significantly older continental crust, and are believed to reflect crustal accretion along convergent plate boundaries³². The Damara granitic rocks exhibit an inverse correlation between initial Nd- and Sr-isotopes (Fig. 4), although the alaskites and the metasediment are displaced to comparatively high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios consistent with a relative increase in Rb/Sr at higher levels in the continental crust.

We thank M. P. Coward, C. J. Hartnady, J. Barnes, K. Downing and A. G. Marlow for discussions and A. R. Gledhill, J. C. Roddick and A. Kröner for unpublished results and comments, and also Professor Nicolaysen for his constructive review. Analyses were carried out at Leeds where isotope and REE research is funded by NERC and the Royal Society.

Received 9 June; accepted 20 November 1980.

1. O'Nions, R. K., Evensen, N. M. & Hamilton, P. J. *Phil. Trans. R. Soc.* (in the press).
2. DePaolo, D. P. & Wasserburg, G. J. *Geochim. cosmochim. Acta* **43**, 615–627 (1979).
3. Hawkesworth, C. J., Norry, M. J., Roddick, J. C. & Vollmer, R. *Nature* **280**, 28–31 (1979).
4. Hawkesworth, C. et al. *Earth planet. Sci. Lett.* **42**, 45–57 (1979).
5. Hawkesworth, C. J. in *Origin of Granite Batholiths: Geochemical Evidence*, 76–89 (Shiva, Kent, 1979).

6. Jacobsen, G. B. & Wasserburg, G. J. *Earth planet. Sci. Lett.* **41**, 245–253 (1978).
7. Hamilton, P. J., Evensen, N. M., O'Nions, R. K. & Tarney, J. *Nature* **277**, 25–28 (1979).
8. McCulloch, M. T. & Wasserburg, G. J. *Science* **200**, 1003–1011 (1978).
9. Kennedy, W. Q. *8th A. Rep. Res. Inst. Afr. Geol.* **48** (University of Leeds, 1964).
10. Kröner, A. *Tectonophysics* **40**, 101–135 (1977).
11. Martin, H. & Porada, H. *Precamb. Res.* **5**, 311–338 (1977).
12. Blaxland, A., Gohn, E., Haack, U. & Hoffer, E. *Neues. Jb. Miner. Mh.* **11**, 498–508 (1979).
13. Coward, M. (in preparation).
14. Watters, B. R. *Nature* **259**, 471–473 (1976).
15. Malling, S. A. *Rep. Precamb. Res. Unit* Vol. 15, 183–193 (University of Cape Town, 1978).
16. Jacob, R. E., Kröner, A. & Burger, A. J. *Geol. Rdsch.* **67**, 706–718 (1978).
17. Burger, A. J., Clifford, T. N. & Miller, R. McG. *Precamb. Res.* **3**, 415–431 (1976).
18. Burger, A. J. & Walraven, F. *Annls. Geol. Surv. S. Afr.* **11**, 323–326 (1978).
19. Burke, K., Dewey, J. F. & Kidd, W. S. F. *Tectonophysics* **40**, 69–99 (1977).
20. Miller, R. McG. *Geol. Surv. S. Afr. Mem.* **64** (1973).
21. Kröner, A. & Hawkesworth, C. J. *Rep. Res. Inst. Afr. Geol.* Vol. 20, 14–16 (University of Leeds, 1977).
22. Mitchell, R. H. & Brunfelt, A. O. *Contr. Miner. Petrol.* **52**, 247–259 (1975).
23. Wendlandt, R. F. & Harrison, W. J. *Yb. Carnegie Inst. Wash.* **77**, 695–703 (1978).
24. Nance, W. B. & Taylor, S. R. *Geochim. cosmochim. Acta* **40**, 1539–1551 (1976).
25. Shaw, D. M., Dostal, J. & Keays, R. R. *Geochim. cosmochim. Acta* **40**, 73–83 (1976).
26. Sawyer, E. W. thesis Univ. Cape Town (1978).
27. Condie, K. C. *Chem. Geol.* **21**, 131–149 (1978).
28. Berning, J., Cooke, R., Hiemstra, S. A. & Hoffman, U. *Econ. Geol.* **71**, 351–368 (1976).
29. DePaolo, D. J. & Wasserburg, G. J. *Geophys. Res. Lett.* **4**, 465–468 (1977).
30. O'Nions, R. K., Carter, S. R., Cohen, R. S., Evensen, N. M. & Hamilton, P. J. *Nature* **273**, 435–438 (1978).
31. Arth, J. G. & Hanson, G. N. *Geochim. cosmochim. Acta* **39**, 325–362 (1975).
32. Gass, I. G. J. *geol. Soc. Lond.* **134**, 129–138 (1977).

A model for Plinian eruptions of Vesuvius

M. F. Sheridan*, F. Barberi, M. Rosi & R. Santacroce

Istituto di Mineralogia e Petrografia, via S. Maria 53,
56100 Pisa, Italy

The term 'Plinian' has been widely used^{1–4} to describe continuous gas-blast eruptions of large magnitude a typical example⁵, of which is the AD 79 eruption of Vesuvius which destroyed Pompei and the surrounding region. We develop a new model here for the AD 79 event that explains the complete Plinian eruptive episode including pyroclastic fall, pyroclastic flow, base surge, laharc and phreatic activity. This model has widespread implications with regard to volcanic hazard evaluation and geothermal exploration at Vesuvius and other volcanoes with similar patterns of activity, such as Mount St Helens.

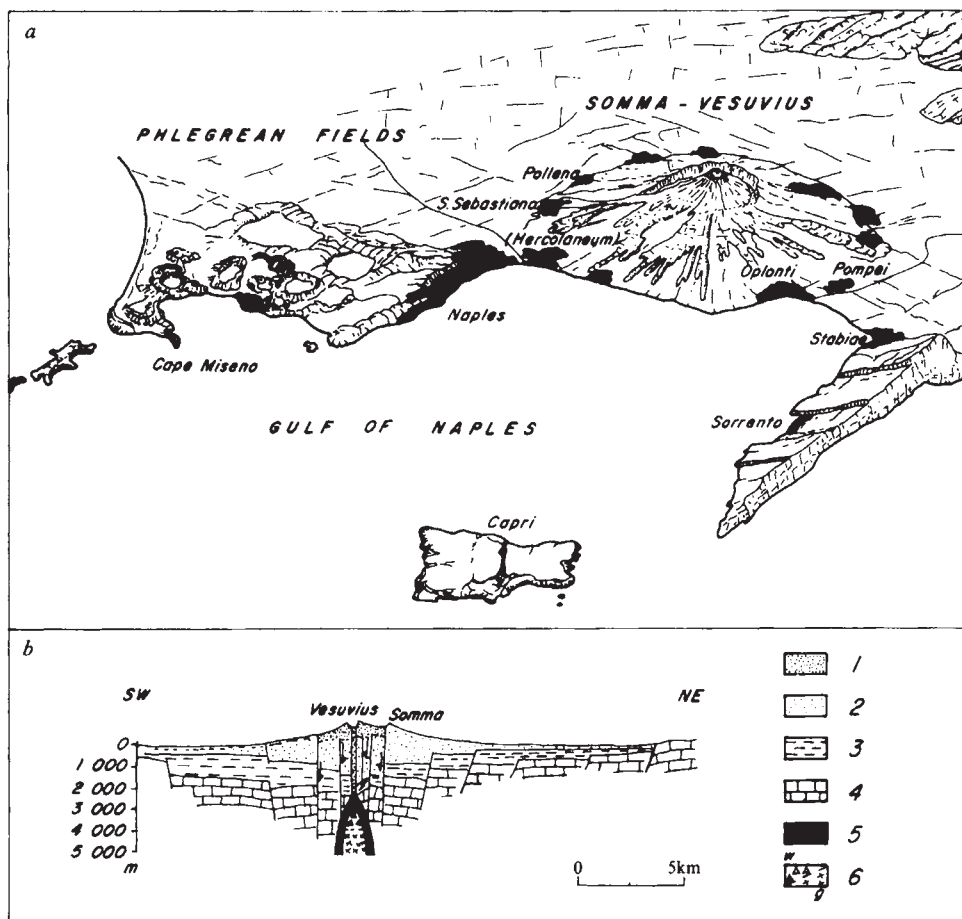
Three representative sections from archaeological excavations demonstrate the principal characteristics of the entire eruptive sequence, assuming emplacement of pyroclastic flows, surges and lahars above the pumice bed, during the 30-h eruptive period. Data from more than 30 sections included in the new model show that factors such as distance from the vent, topography and wind velocity, control the depositional facies.

At the Pompei excavation 4.5-m of section (Fig. 2) includes a lower white pumice-fall layer that grades into an upper grey, pumice-fall bed overlain by interbedded surge and grey pumice-fall levels and an uppermost surge sequence. There is an upward increase in lithic xenoliths from 12 wt% for the white part to 20 wt% for the grey⁵. The character of the xenoliths also changes with a slight increase in fraction of carbonates and a strong increase in cognate cumulates in the grey level. The lower group of surge beds is of the dry (superheated steam) massive and sandwave bed type^{9,10}. This group includes two prominent phreatic fall levels rich in scoria and calcareous fragments. The contact between the grey pumice and the first surge beds is a prominent wave form. The uppermost part of the surge sequence probably represents emplacement of very wet (condensed steam) phreatomagmatic products. These massive fine-ash beds are full of accretionary lapilli and may have the widespread dispersal characteristic of phreatoplinian deposits¹¹.

At the Oplonti excavation the section is 8.5-m thick (Fig. 2). The grey pumice-fall layer, which is much thicker than the white,

* Present address: Department of Geology, Arizona State University, Tempe, Arizona 85281.

Fig. 1 Somma-Vesuvius Volcano. *a*, Location map. *b*, Schematic cross-section. Key: 1, volcanic rocks of Vesuvius; 2, volcanic rocks of Somma; 3, Tertiary and Quaternary clastic sediment; 4, Mesozoic limestone; 5, chilled margin and contact metamorphic halo of the magma chamber; 6, differentiated magma of the AD 79 magma chamber that produced the white (w) and grey (g) pumice of the Plinian eruption.



is interrupted by three prominent surge beds. The progressive strength of the surge blasts is indicated by the increase in cast diameter of destroyed trees from 10 cm for the lower surge to 20 cm for the upper one. Above the grey pumice is a prominent sandwave horizon that contains a broken tree trunk 25 cm in diameter, and a mould of a tree 40 cm in diameter. Above the surge sequence are two pyroclastic flows. The lower one is rich in grey pumice and lacks stratification, the upper one has

numerous reversely graded laminations, probably indicating that it is at its distal reaches¹². Above the pyroclastic flows is a series of wet surge deposits with planar and massive beds rich in pisolites.

The Herculaneum section is ~20 m thick (Fig. 2). There is no pumice-fall horizon: at the base are sandwave surge beds. Above is a pumice-rich pyroclastic flow containing fragments of houses, carbonized wood and fumarolic pipes towards the top.

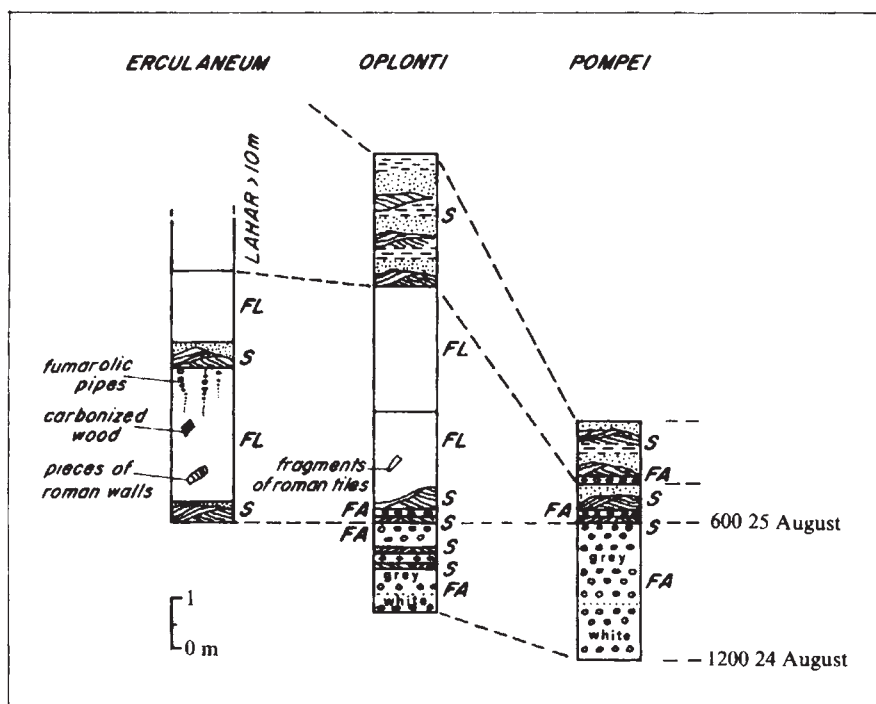


Fig. 2 Representative sections of AD 79 pyroclastic deposit from archaeological excavations. FA, air fall pumice; S, surge deposits; FL, pyroclastic flows.

The overlying sequence of sandwave surge deposits is followed by another pyroclastic flow. The remainder of the section is composed of lahars.

These deposits can be related to eruption phenomenology. A large volume ($\sim 2 \text{ km}^3$) of tephritic magma intruded a shallow (3–5 km deep) magma chamber within the Mesozoic limestone series^{6–8}. The residence time of the magma was sufficient for a reaction skarn to form and a contact metamorphic halo that extended outwards into the carbonate country rocks to develop⁷. The metamorphic halo effectively sealed the differentiating magma system, although p_{CO_2} may have been significantly increased within the chamber as a result of decarbonation reactions in the carbonate wall rocks. A chilled magma margin may have originally formed, but with slow cooling and crystallization a coarser-grained syenite layer grew inwards from the upper part of the chamber as mafic cumulates settled to the bottom. The long period of differentiation produced a stagnant compositionally zoned upper zone of evolved magma rich in volatiles. The basic lower part was more homogenous, presumably due to circulation.

The eruption began (Fig. 3a) either when the gas pressure within the chamber became sufficient to rupture a conduit to the surface or, less likely, with the introduction of a new batch of hot magma from depth¹³. The initial eruption followed the model of Wilson^{14–16} with decompression due to exsolving magmatic gas driving the gas thrust during the opening and widening of the conduit. Buoyant convective uplift of particles above the continuous gas thrust zone produced a persistent eruptive column from which the Plinian pumice-fall deposits were derived through lateral wind shear¹⁷. Using the average wind velocity at 9–11 km elevation (20 m s^{-1}) and terminal fall velocity data⁵, a column height of $\sim 17 \text{ km}$ is computed. This corresponds well with the height of 16–26 km calculated from the theoretical formulae of Wilson *et al.*¹⁶, assuming an average volume eruption rate of $4 \times 10^4 \text{ m}^3 \text{ s}^{-1}$ for an 18-h period and an F value of 0.3–1.0.

The initial eruption produced the white highly fractionated grey pumice-fall with $\sim 10 \text{ wt\%}$ lithic clasts in the approximate ratio of the stratigraphy traversed by the conduit. The eruption proceeded uninterrupted into the less-fractionated grey pumice-fall level with an increase in lithics and fraction of carbonates relative to other lithics and the appearance of felsic cumulates. This represents the removal of solidified magma and country rock surrounding the upper part of the magma chamber.

The greater density, larger size and wider dispersal of the grey pumice indicate a greater column height than for the white pumice. Lirer *et al.*⁵ attribute this to tapping of a long (5 km) narrow (340 m diameter) magma chamber to provide sufficient increase in gas pressure to offset the decrease in gas content. The present data which indicate a magma chamber of $\sim 2 \text{ km}$ height and 1 km diameter, cast doubt on the above mechanism. Rather, the increased explosivity which widens the vent conduit system above the chamber could result from vaporization of a small mass ratio of external water to magma in the chamber during the grey pumice eruption. Minor surge layers within the upper part of the grey pumice deposit at Oplonti and other locations and an increase in abundance of lithic inclusions in the grey pumice support this interpretation.

The initial mass fraction of xenoliths in the white pumice was small and their ratio was proportional to their relative abundance in the stratigraphic column above the chamber. As the chamber became partially emptied during the eruption of the grey pumice, cavitation of the roof and the walls took place, (Fig. 3b) as indicated by the sharp increase in the proportion of deep inclusion types such as high-grade contact metamorphics, skarns and cognate plutonic nodules. The eruption may have become discontinuous at this stage.

Rupturing of the tight metamorphic encasement allowed interaction with the regional hydrologic system (Fig. 3c). Because the magma level within the emptying chamber was below the principal aquifer (Mesozoic carbonate sequence) an abundant source of water was available to the chamber when the

venting pressure dropped below that of the hydrostatic head ($\sim 300\text{--}500 \text{ bar}$). The immediate effect was to generate strong hydromagmatic or phreatic explosions, depending on the degree of mixing of water and magma before decompression of the steam. The products included surges of fine-grained ashes, widespread phreatoplinian layers, vesiculated tuffs¹⁸ and phreatic breccias.

At this stage, the production of small pyroclastic flows may have been directly related to contemporaneous hydromagmatic and magmatic activity occurring in different parts of the chamber. Marginal hydromagmatic explosions would have provided the fine fragmentation typical of pyroclastic flows^{19–21} that is difficult to explain by other processes such as column collapse. Magmatic eruptions from the centre of the chamber would have produced the coarse pumice and provided a strong gas blast to maintain the eruption column. Homogenization within the chamber, vent and eruption column accounts for the rounding of the pumice and uniform texture characteristic of the pumice flows. Cooling of the system by water vaporization and enlargement of the vent due to strong explosions would have favoured column collapse.

At first hydromagmatic explosions alternated with purely magmatic activity; later they dominated. Because the eruptive system was gradually cooled through water vaporization during

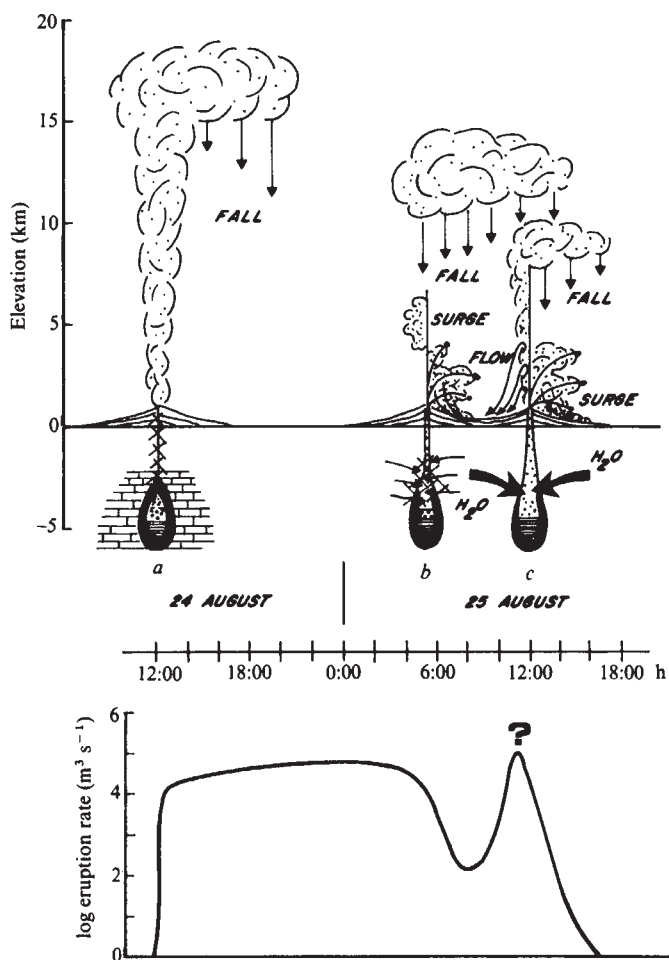


Fig. 3 Model of the AD 79 eruption of Vesuvius related to eruption phenomenology described by Pliny the Younger²³. *a*, Gas pressure in the chamber ruptures a path to the surface producing an eruption column of about 17 km height. The eruptive rate during this phase averages $4.0 \times 10^4 \text{ m}^3 \text{ s}^{-1}$. *b*, A strong reduction in activity associated with cavitation of the chamber roof produces intermittent magmatic and hydromagmatic explosions. *c*, The terminal phase is dominantly hydromagmatic with pyroclastic flows and surges as the products. The eruption rate may reach $10^5 \text{ m}^3 \text{ s}^{-1}$, close to the maximum theoretical rate for a prolonged maintained column²⁰.

the hydromagmatic phase, the products proceed through early hot dry surges and pyroclastic flows which are followed by colder wet surges and lahars and finally by phreatic explosion breccias. If the chamber had become completely evacuated of magma before significant hydromagmatic cooling, the final eruption would have been purely phreatic. These later products included xenoliths of syenite and mafic cumulates of biotite, olivine and pyroxene torn from the walls and floor of the chamber.

A similar pattern has been observed in four other Plinian eruption cycles identified at Vesuvius²²: Avellino (3,500 yr BP), Gemelle (8,000 yr BP), Verdoline (13,000 yr BP) and Pomici di Base (17,000 yr BP). All began with a highly fractionated pumice-fall deposit in which the percentage of lithics increases with time. Those eruptions characterized by strong hydromagmatic activity (pyroclastic flow and surge) had a strong concentration of carbonate lithics in the pumice-fall immediately below the surge beds. This indicates an enlargement of the

chamber roof within the regional aquifer by removal of the surrounding country rock. The surge deposits proceeded through an early hot dry type associated with pyroclastic flows to a final cold wet or even phreatic type.

The late-stage behaviour of the eruption depends both on internal magma dynamics, and on the hydrological framework. For example, the Verdoline magma chamber was located in the relatively impervious Tertiary flysch marls above the Mesozoic carbonate basement. Although there was an increase in amount of lithic clasts with time in the pumice fall deposit, the hydromagmatic phase was only minor. The other Plinian eruptions with magma chambers entirely within the carbonate basement, to the contrary, have produced extensive hydromagmatic and phreatic deposits.

This work was supported by USA-Italy cooperative research program of NSF, grant INT-7823984, and by the Italian Geodynamics Project, publication 319.

Received 6 May; accepted 31 October 1980.

1. Walker, G. P. L. & Croasdale, R. J. *geol. Soc. Lond.* **127**, 17-55 (1971).
2. Walker, G. P. L. *Geol. Rdsch* **62**, 431-446 (1973).
3. Sparks, R. S. J. & Wilson, L. J. *geol. Soc. Lond.* **132**, 441-451 (1976).
4. Nairn, I. A. & Self, S. J. *Volcan. geotherm. Res.* **3**, 39-60 (1978).
5. Lirer, L., Pescatore, T., Booth, B. & Walker, G. P. L. *Bull. geol. Soc. Am.* **84**, 759-772 (1973).
6. Barberi, F. *et al.* *IUGG, XVII General Assembly, Canberra* (1979).
7. Barberi, F. & Leoni, L. *Bull. volcan.* (in the press).
8. Barberi, F. *et al.* *EEG 2nd Seminar on Geothermal Energy, Strasbourg* (1980).
9. Sheridan, M. F. & Updike, R. G. *Bull. geol. Soc. Am.* **86**, 571-581 (1975).
10. Wohletz, K. H. & Sheridan, M. F. *Geol. Soc. Am. Spec. Pap.* **180**, 177-193 (1979).
11. Self, S. & Sparks, R. S. J. *Bull. volcan.* **41**, 196-212 (1978).
12. Sheridan, M. F. *Geol. Soc. Am. Spec. Pap.* **180**, 125-136 (1979).
13. Sparks, R. S. J., Sigurdsson, H. & Wilson, L. *Nature* **267**, 315-318 (1977).
14. Wilson, L. *Geophys. J. R. astr. Soc.* **30**, 381-392 (1972).
15. Wilson, L. *Geophys. J. R. astr. Soc.* **45**, 545-556 (1976).
16. Wilson, L., Sparks, R. S. J., Huang, T. C. & Watkins, N. D. *J. geophys. Res.* **83**, 1829-1836 (1978).
17. Walker, G. P. L., Wilson, L. & Boswell, E. L. *Geophys. J. R. astr. Soc.* **22**, 377-383 (1971).
18. Lorenz, V. *Sedimentology* **21**, 273-291 (1974).
19. Walker, G. P. L. *J. Geol.* **79**, 696-714 (1971).
20. Sheridan, M. F. *J. geophys. Res.* **76**, 5627-5634 (1971).
21. Sparks, R. S. J. *Sedimentology* **23**, 147-188 (1976).
22. Delibrias, G., Di Paola, G. M., Rosi, M. & Santacroce, R. *R. Soc. Ital. Miner. Petrol.* **35**, 411-438 (1979).
23. Radice, B. *Pliny Letters and Panegyricus I* (Harvard University Press, Massachusetts, 1972).

Middle Ordovician chondrite in fossiliferous limestone from Brunflo, central Sweden

Per Thorslund

Department of Palaeontology, Uppsala University, Box 558,
S-751 22 Uppsala, Sweden

Frans E. Wickman

Department of Geology, University of Stockholm, Box 6801,
S-113 86 Stockholm, Sweden

The few discoveries of fossil meteorites have been attributed¹ to the ignorance about the appearance of meteorites among palaeontologists and other geologists who examine limestone quarries, coal mines, and other excavations in sedimentary rocks. We now report the first find of a fossil stony meteorite in Ordovician limestone. The meteorite is a chondrite, possibly an H-chondrite, and its terrestrial age is ~463 Myr. The fine structural details of the chondrules are often extremely well preserved, but the chemical composition and the mineralogy have changed dramatically, chromite being the only primary mineral preserved. The present major minerals are calcite, barite, a Cr-V- 'phengite' and a cobaltite-group mineral.

An earlier find reported by Yudin² consists only of isolated barred chondrules in a Mesozoic bauxite. In 1952, a polished slab of Ordovician limestone was sent to one of us (P.T.) for examination because it contained an almost black clast about 10 cm across (Fig. 1). Thin sections were prepared of the foreign body and were examined by a petrographer, who reported that the clast was a metamorphic ultramafic rock consisting mainly of clinozoisite, calcite, magnetite and serpentine. This unusual discovery was explained by algae transporting the stone to its

present position. However, as no similar ultramafic rocks were known on the continental (eastern) side of the Brunflo area the slab was set aside. The Siljan Ring, over 200 km to the south of Brunflo was one of Sweden's best areas for Ordovician and Silurian rocks, is an impact structure. In December 1979 the slab

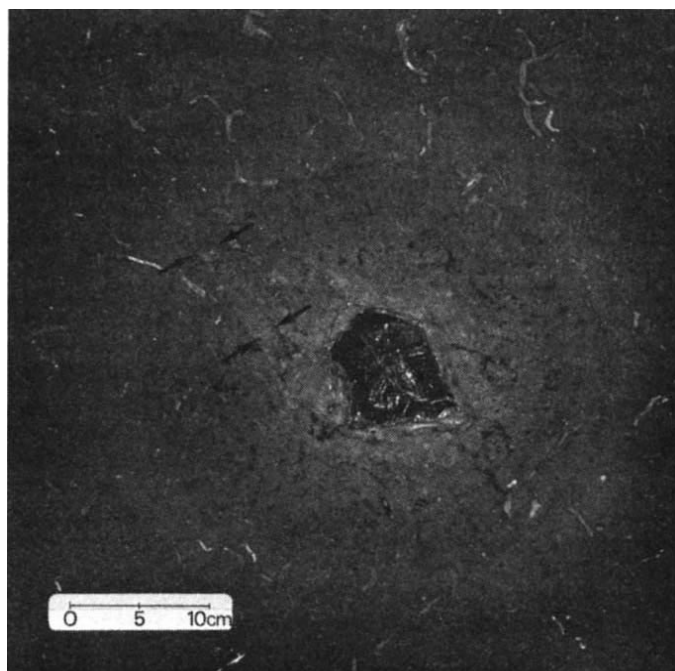


Fig. 1 The polished slab of limestone with the chondrite. The size of the slab is ~65 × 65 cm, and the meteorite ~10 cm across. The dark portions contain the structurally best preserved chondrules. The white portions are now calcite and barite. The cephalopod (arrowed) seems to have been struck by the meteorite. The differently coloured zones around the meteorite are visible.

Table 1 Electron microprobe analyses of chondritic chromites

	1	2	3	4	5	6	7	8	9
Cr ₂ O ₃	56.8	56.9	56.1	54.4	55.9	60.8	57.2	56.7	57.2
Al ₂ O ₃	6.5	5.9	5.3	5.7	6.0	3.9	6.3	5.9	5.8
V ₂ O ₃	0.74	0.68	0.72	0.73	0.65	0.70	0.71	0.68	0.69
TiO ₂	1.73	2.33	2.81	3.23	2.72	1.47	2.12	2.27	2.47
FeO	30.9	31.2	33.0	34.5	30.1	30.5	30.6	31.3	30.8
MgO	1.98	2.66	1.99	1.62	2.72	2.32	2.73	2.51	3.0
MnO	0.50	0.94	0.74	0.63	1.03	0.79	0.86	0.99	0.81
ZnO	0.93	—	—	—	1.09	—	—	—	—
Total	100	100.61	100.66	100.81	100.21	100.48	100.52	100.35	100.77

Analyses 2–9 from ref. 4. Number in parenthesis gives number of meteorites analysed for 2–4, and number of grains analysed for 1, 5 and 6–9.

Analyses: 1, average present find, normalized to a sum of 100 after exclusion of 0.33 wt% CaO (11); 2, average H5 and H6 (10); 3, average L5 and L6 (7); 4, average LL5 and LL6 (6); 5, average Pultusk (4); 6, average H3 (12); 7, average H4 (21); 8, average H5 (28); 9, average H6 (11).

with its clast was re-examined with the idea that it could be a stony meteorite; the presence of chondrule-like boundaries was noticed.

The meteorite occurs in a reddish brown limestone from the Rödbrottet quarry (lat 63°7' N, long 14°17' E), near Brunflo, not far from Östersund, central Sweden. Determination of the conodonts in the slab shows that the meteorite fell during the Aseri Stage of the Middle Ordovician (~463 Myr ago).

In a ~3 cm-wide border zone, delicate structural details of chondrules are preserved, although the primary minerals have been replaced. Two examples are shown in Fig. 2, one barred chondrule and one radiating chondrule. The chondrules are well defined in a matrix, giving the impression of the petrologic types 3–5 of Van Schmus and Wood³.

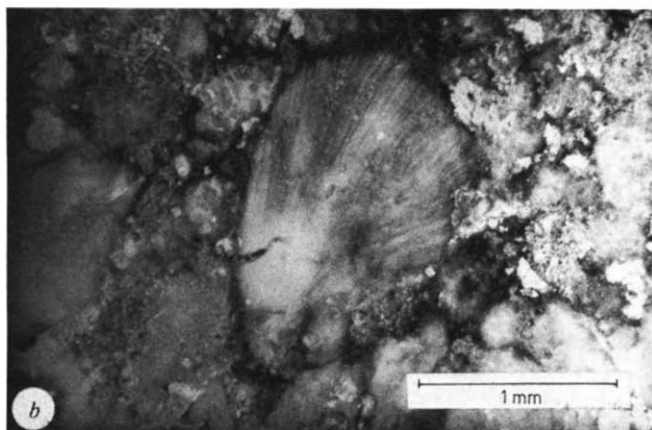
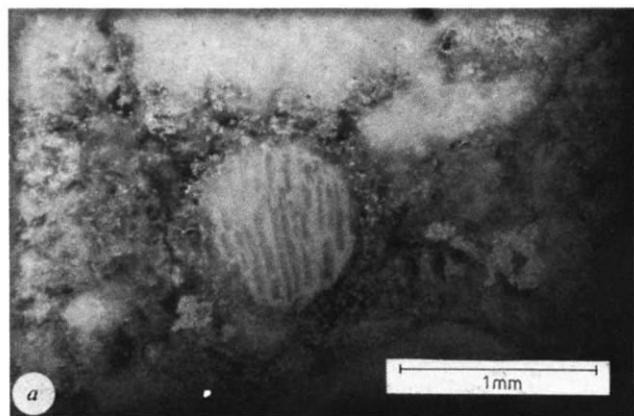


Fig. 2 Examples of well preserved chondrules from the polished surface of the slab: a, a barred chondrule; b, a radiating chondrule.

The present mineralogy of the chondrite is quite different from the original one (and from that given by the petrographer). Olivine, pyroxene, nickel-iron and troilite no longer exist. In fact iron and magnesium have been lost almost completely from the meteorite. Instead, the dominating mineral is calcite, the other main constituents being barite, a Cr–V-bearing 'phengite' and a cobaltite-group mineral with more cobalt than nickel. Electron microprobe experiments reveal that only one primary mineral seems to have been preserved, chromite.

Table 1 shows some chromite analyses. The present chromite is seen to be a typical chondritic chromite, with a homogeneous composition. The calcium content is interpreted as belonging to the calcite. The present chromite contains 0.93 wt% ZnO; the only chondrite found by Bunch *et al.*⁴ to contain zinc-bearing chromite is the H5 Pultusk. It is very difficult to make a definite classification of the find, but it seems to be most similar to the H group, most likely the subgroups H4–H5. However, note that analysis 1 in Table 1 could be interpreted to indicate an unknown group of chondrites.

Around the meteorite are concentric rings in different shades of brown, red, grey and green. They clearly represent reactions between the carbonate mud or, if later, limestone and the original meteoritic matter. These zones have not as yet been studied.

A badly preserved orthocone nautiloid cephalopod, which occurs in contact with the meteorite, could have been hit and killed by the meteorite.

Much of the discussion of the sources of chondritic meteorites focuses on the part played by Apollo and Amor objects in their relationships to comets. Wetherill's results⁵ indicate that for bodies in Earth-crossing orbits one half is eliminated in the first 10 Myr but that the rate of elimination then slows down greatly. He has shown that in steady state conditions and a population of ~800 Apollos (15 added per Myr) their median age will be ~200 Myr and ~35% of them will be more than 500 Myr old. Even if these figures were considered with great caution the present find is not inconsistent with Wetherill's conclusions.

The concentration of cosmic ray exposure ages around 4 Myr for modern H chondrites—this indicates that they come from another source than did the clast we have studied. Of course, it has been assumed that the composition of the chromite corresponds to those of H chondrites and not to some unknown group. A detailed description of this first definite fossil stony meteorite will be given elsewhere⁶.

Received 29 September; accepted 2 December 1980.

1. Ninninger, H. H. *Find a falling star* (Eriksson, New York, 1972).
2. Yudin, I. A. *Meteoritics* **6**, 99–103 (1971).
3. Van Schmus, W. R. & Wood, J. A. *Geochim. cosmochim. Acta* **31**, 747–765 (1967).
4. Bunch, T. E., Keil, K. & Snetsinger, K. G. *Geochim. cosmochim. Acta* **31**, 1569–1582 (1967).
5. Wetherill, G. W. *Yb. Carnegie Instn. Wash.* **77**, 456–471 (1978).
6. Thorslund, P. & Wickman, F. E. *Lithos* (in preparation).

Direct use of dissolved organic carbon by agglutinated benthic foraminifera

T. E. DeLaca*‡, D. M. Karl† & J. H. Lipps*

* Department of Geology, University of California, Davis, California 95616

† Department of Oceanography, University of Hawaii at Manoa, 2525 Correa Road, Honolulu, Hawaii 96822

Foraminifera are known to obtain nutrients in a variety of ways: they are omnivores, carnivores or herbivores¹⁻³ and some species are known to use the extracellular metabolites of their photosynthetic endosymbionts^{1,2,4}. None, however, has previously been proven to utilize exogenous dissolved organic carbon directly, although this is well known in other marine species^{5,6}. Knowledge of foraminiferal trophic positions is important because foraminifera are common in most marine communities¹ and may be the most abundant eukaryotic organism in the extensive deep-sea benthos⁷⁻⁹. Our studies of benthic foraminifera from an unusual Antarctic shallow water embayment now show that certain species utilize both particulate and dissolved organic material in their nutrition.

Our studies were conducted at McMurdo Sound, Antarctica, located in the southwest corner of the Ross Sea at latitude 77°S and longitude 166°E (Fig. 1). The Sound is approximately 50 km wide; however, its eastern and western sides differ greatly in water column productivity and benthic biomass due to local oceanographic conditions¹⁰ (see Fig. 1). The eastern side of McMurdo Sound is extremely productive during the austral summer, whereas the western side at New Harbor has very low productivity. The western side is rich in nitrate, phosphate and silica and low in plankton and particulate organic material (ref. 11 and D.M.K., unpublished data). Based on water analyses, species diversity and faunal affinities, New Harbor is more similar to the deep sea than to other shallow-water areas around Antarctica¹¹. The organisms we discuss here were found only at New Harbor. Our investigation covered an area of 15–40 m depth, with a temperature of -1.8°C . The sediments were rich in organic material. Analyses of dissolved amino acids, as shown by fluorometric analyses¹² of water collected in dialysis bags, demonstrated maximum concentrations in the sediment (20–30 μM at 1 cm) and smaller concentrations in the water column (5 μM at the sediment water interface, 1 μM at 10 cm from the bottom). This trend in the distribution of amino acids is similar to that of total dissolved organic carbon in the deep sea¹³.

Our studies focused on *Notodendrodes antarctikos*¹⁴ and an as yet undescribed species. Both are arborescent agglutinated foraminifera in the superfamily Ammodiscacea (Fig. 2). These large foraminifera are conspicuous components of the bottom assemblages at New Harbor, with abundances of >200 individuals per m^2 (ref. 14). Our initial studies on the physiology and trophic positions of these organisms revealed that they use captured suspended particulate organic material and dissolved organic materials in their nutrition. As seen by scanning electron microscopy of specimens which were fixed *in situ* and later dried using a critical-point technique, suspended particles are trapped in sticky cytoplasmic masses which extend from the branches of the tests. The foreign material caught in pseudopodia consists of benthic diatoms, bacteria, amorphous flocculent organic and inorganic material (such as silt, sand, broken diatom frustules and sponge spicules) with adhering bacteria. This material is probably suspended by the activities of larger invertebrates. Marine bacteria, labelled with ^3H -adenine (in the laboratory) were presented to foraminifera *in situ*, and these foraminifera were later fixed *in situ*. Microautoradiography revealed that the

labelled bacteria are captured, digested and incorporated into the protoplasm of the organisms. However, the capture of particulate matter, in natural conditions, seems to occur infrequently in the population studied (3 of 32 specimens).

The foraminifera were tested for their ability to take up dissolved organic carbon by exposing them to several radiolabelled substrates, including amino acids (algal protein hydrolysate, Amersham), glucose and adenine (Table 1). We found that they took up the first two but did not absorb adenine. As bacterial contamination in our experiments could have produced erroneous results, we used ^3H -adenine (rapidly taken up by most bacteria¹³) in some of our experiments as a control in dual-label experiments with ^{14}C -labelled substrates. When tritium was detected in liquid scintillation analyses, those samples were assumed to be contaminated and were eliminated. 'Time zero' and NaCN-killed controls were used in all experiments.

Experiments measuring the uptake of dissolved organic carbon were conducted in the laboratory. Foraminifera cleaned in filter-sterilized sea water (0.22 μm) were placed in 10 ml of filtered (0.22 μm) sea water (1.0 μm free amino acids in solution) containing 2.5 μCi of ^{14}C -UL-protein hydrolysate

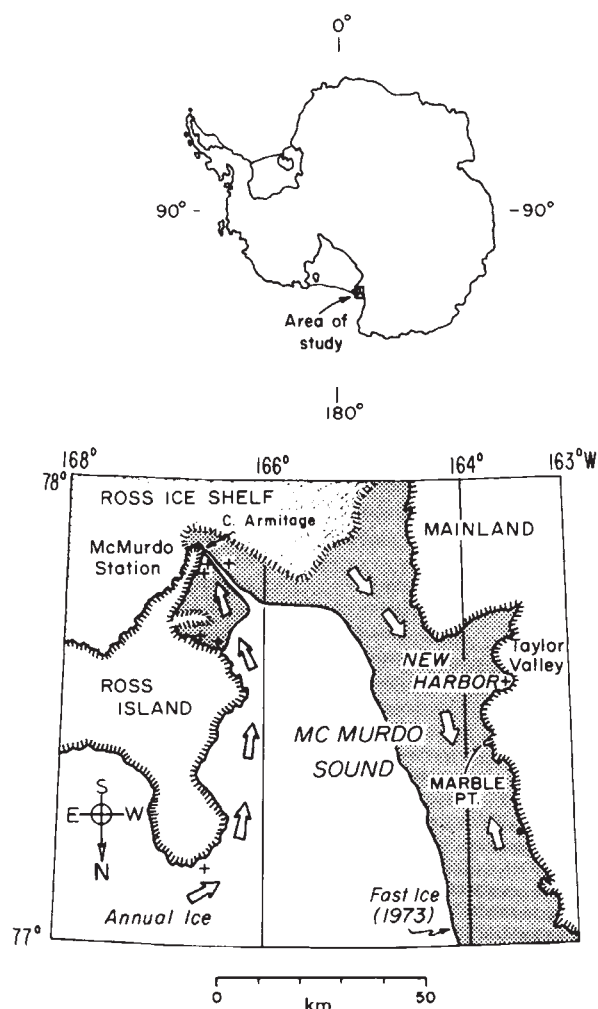


Fig. 1 Antarctica, showing the location of the study area. [During past investigations in McMurdo Sound we have surveyed several localities (+).] The two new species of foraminifera discussed in the present report were found only at New Harbor. Arrows indicate the probable direction of water current movement. In the eastern and central parts of the Sound currents move south, advecting large standing crops of plankton from more northerly waters. The annual break up of sea ice on the eastern side allows local productivity to increase dramatically. In contrast to the highly productive eastern side, the western side of the Sound is bathed by northward moving currents¹² which probably originate under the Ross Ice Shelf and are poor in organic nutrients.

‡ Present address: Marine Biology Research Division A-002, Scripps Institution of Oceanography, La Jolla, California 92093.

(0.0165 μmol free amino acids). The final concentration of free amino acids in solution was 2.65 μM , and incubation times of 0.58, 1.08, 2.25, 3.25 and 4.17 h were used, all experiments being conducted at -1.0°C . Following incubation, the foraminifera were washed and: (1) solubilized in hot and cold 10% trichloroacetic acid (TCA), (2) placed in sealed reaction flasks with 10 ml of filtered sea water for measurement of $^{14}\text{CO}_2$ evolution, or (3) fixed for autoradiography.

Cellular contents of solubilized foraminifera were analysed using liquid scintillation. Uptake rates of $6.14 \times 10^{-4} \mu\text{mol h}^{-1}$ (s.d. = 4.26×10^{-4} , $n = 20$) were found. The tests of individual organisms were subsequently removed, washed three times in hot and cold 10% TCA to remove any remaining soluble material and re-analysed. Up to 41.3% of the label was incorporated into non-soluble material that remained in the test.

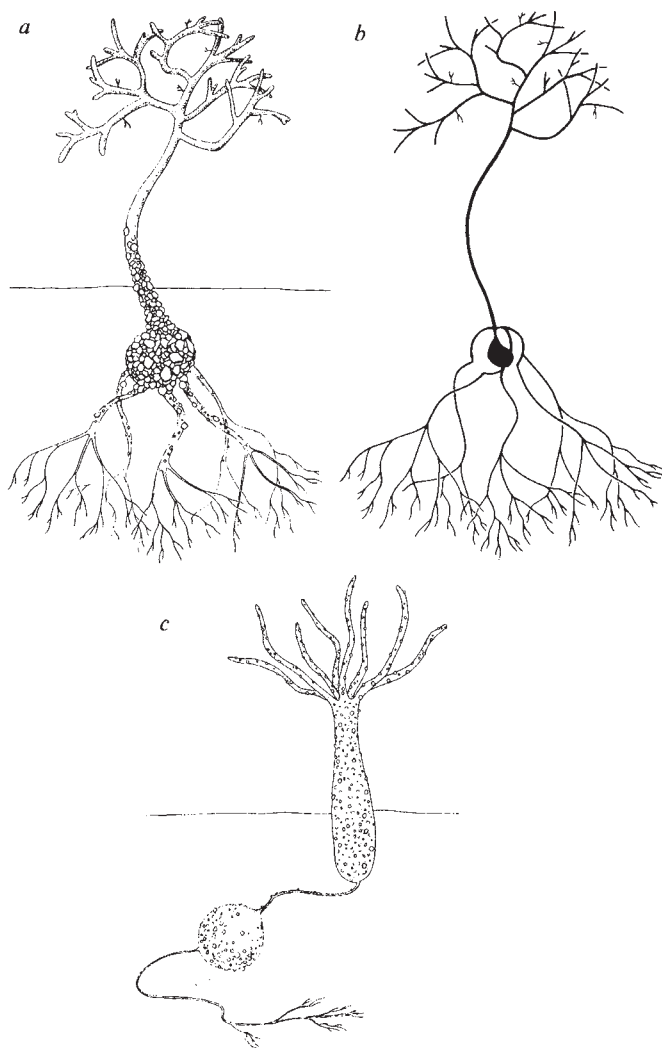


Fig. 2 *a*, *Notodendrodes antarctikos*¹⁴ drawn in life position. The branching upper half extends above the sediment-water interface and the bulb and root system are buried in the sediment (average length 20 mm). *b*, The protoplasm of this species as would be seen if the test was removed. Cytological studies have shown that approximately one-third of the protoplasm (containing the nucleus) resides within the bulb. About one-third of the cytoplasm extends up the stem and occupies the branching system. Small pseudopods extend from the branches and upper portions of the stem. Particulate organic material is entrapped in these web-like projections. The remaining one-third of the cytoplasm resides in the extensive root system. Autoradiographic studies have shown that although dissolved organic material is taken up on all exposed cytoplasmic surfaces, the highest uptake occurs in the root system where the surface area is greatest. *c*, An unnamed agglutinated foraminifer (*Notodendrodidae* gen. et sp. n.) drawn in life position (average size 18 mm).

Table 1 Summary of uptake

Protein hydrolysate	
Total uptake	6.14 $\mu\text{mol h}^{-1}$
Incorporation into TCA-insoluble form	41.3% h^{-1}
$^{14}\text{CO}_2$ liberated in 12 h	10%
Glucose	
Total uptake ^{14}C -glucose	$6.1 \times 10^{-2} \mu\text{mol h}^{-1}$
$^{14}\text{CO}_2$ liberated in 12 h	46%
Adenine	
Total uptake	Undetectable

Experiments were conducted at -1°C . Final concentration of free amino acid in laboratory experiments was 2.65 μM . Concentration of free amino acid in the sediment where the organisms are found was found to be 20–30 μM . See text for further explanation. Concentrations of glucose in the environment and laboratory experiments were not adequately measured.

The labelled foraminifera placed in reaction vials were incubated for 12 h at -1°C . The incubation was terminated with the addition of 0.2 ml of ethanolamine (placed on filter paper in the reaction vessel) and 1 ml H_2SO_4 (0.5 M) was injected into the seawater. After 2 h at room temperature (23°C), the ethanolamine and filter paper were analysed by liquid scintillation. The results demonstrated that 10.0% of the total ^{14}C taken up was respired.

The results of autoradiography showed that the foraminifera took up dissolved organic carbon through any cell surface; consequently, uptake was a function of surface area. The 'root system', with its large surface area, was the principal site of uptake in the organisms analysed.

These two species of foraminifera seem to have ecological and physiological adaptations which are well suited to a highly seasonal oligotrophic environment. At New Harbor, primary productivity is very low and limited to a period of 1 or 2 months during the year, during which time the growth of a diatom flora on the undersurface of the 3–4-m thick sea ice or on the sediments of very shallow water (<10 m) provides sparse organic input. The under-ice flora rains down to the bottom, where a portion is directly utilized while the remainder enters the detritus food web. Decomposition of this sedimented organic material generates relatively high concentrations of dissolved organic carbon in the sediment and supports a large and metabolically active microbial population (D. M. K., unpublished data). These foraminifera can capture and digest this particulate material either as it settles to the bottom or as it is resuspended by deposit-feeding invertebrates, although capture of particles seems to occur only infrequently. Dissolved organic carbon may contribute significantly to the nutrition of these foraminifera. During periods of no primary productivity, ~10 months of the year, they may sustain themselves on the high dissolved organic carbon reserves which are continually generated by the activities of heterotrophic microorganisms in the sediment.

Our results demonstrate that foraminifera are able to use both saprozoic and holozoic (phagotrophic) modes of nutrition. The amount and type of organic material available to foraminifera are therefore both much greater and more diverse than was previously thought. Re-evaluation of current ideas of the trophic positions of these extremely abundant organisms in benthic ecosystems is necessary, especially in deep-sea communities where they have been found to represent the highest test-free biomass of all eukaryotic organisms⁹.

Present studies are analysing the metabolic rates of these organisms and will determine the proportion of their total energy requirements supplied by dissolved organic carbon.

We thank Professor A. E. V. Haschemeyer for her assistance in experimental design and encouragement, Drs W. L. Stockton and R. R. Hessler for reading the manuscript and Ms E. Bailey for drawing the figures. This research was funded by NSF grant DPP 76-17231.

Received 14 April; accepted 24 October 1980.

1. Boltovskoy, E. & Wright, R. *Recent Foraminifera* (The Hague, 1976).
2. Lee, J. J. in *Foraminifera* (eds Hedley, R. H. & Adams, C. G.) 207 (Academic, London, 1974).
3. Lee, J. J. *et al.* *J. Protozool.* **13**, 659–670 (1966).
4. Smith, D. F. & Wiebe, W. J. *Aust. J. Fish. Fresh. Res.* **28**, 311–319 (1977).
5. Jorgensen, C. B. *Biol. Rev.* **51**, 291–328 (1976).
6. Stevens, G. C. in *Symp. Nitrogen Metabolism and the Environment* (eds Campbell, J. W. & Goldstein, C.) 155–184 (Academic, New York, 1972).
7. Hessler, R. R. in *The Biology of the Oceanic Pacific* (ed. Miller, C. B.) 79–93 (Oregon State University Press, Corvallis, 1974).
8. Tendal, O. & Hessler, R. R. *Galathea Rep.* **14**, 165–194 (1977).
9. Smith, K. L. Jr *Mar. Biol.* **47**, 337–347 (1978).
10. Littlepage, J. L. *Antarct. Res. Ser.* **14**, 1–37 (1971).
11. Dayton, P. K. & Oliver, J. S. *Science* **197**, 55–58 (1977).
12. North, B. B. *Limnol. Oceanogr.* **20**, 20–27 (1975).
13. Karl, D. M. *Appl. envir. Microbiol.* **38**, 850–860 (1979).
14. DeLaca, T. E., Lipps, J. H. & Hessler, R. R. *J. Linn. Soc. Lond. Zool.* **69**, 205–224 (1980).

Lichen recolonization in London's cleaner air

C. I. Rose* & D. L. Hawksworth†

* Department of Applied Biology, Chelsea College, University of London, Hortensia Road, London SW10 0QX, UK

† Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey TW9 3AF, UK

Mean sulphur dioxide levels have fallen markedly in London, UK during the past 15 yr. Although lichens growing on trees are particularly sensitive to this air pollutant, there are few studies of lichen recolonization following pollution episodes. A survey of 29 sites in the north and west of Greater London, reported here, demonstrates that several species extinct or very rare in the area in 1970 have extended their ranges considerably. Studies on growth rates of the species concerned suggest that many of the sites discovered have been recolonized within the past 3–7 yr. If current trends continue, further improvements in the lichen flora can be expected in the next few years. However, it is unlikely that London will regain in the foreseeable future many of the species lost during the past two centuries.

In the British Isles, smoke and sulphur dioxide (SO₂) levels have fallen by ~80% and 50% respectively in urban areas since 1960¹; in Inner London, the mean annual level of SO₂ declined from 200–250 µg m⁻³ to <130 µg m⁻³ in the same period². Recorded changes in mean annual and mean winter SO₂ values at six stations in north and west London are shown in Fig. 1. Lichens have been used extensively as indicators of air pollution and have proved to be of particular value in estimating levels of SO₂ pollution in temperate regions^{3–8}. Most studies have been concerned with changes in conditions of increasing levels of air pollutants but there are a few scattered reports of improving lichen vegetation in ameliorating conditions from Germany⁹, Sweden^{10,11} and several urban areas of the British Isles—Birmingham¹², Leeds^{13–15}, Newcastle-upon-Tyne¹⁶ and Sheffield¹⁷. Some increases in the abundance of the SO₂-tolerant *Lecanora conizaeoides* between 1953 and 1967 at Holland Park, London, have been reported¹⁸ and there are a few recent records of other more sensitive species from London, including a single plant of *Hypogymnia physodes* at Brent Reservoir in 1972 (ref. 19), and *Parmelia sulcata* on a wall at Brook Green in 1976 by P. W. James (personal communication). No detailed survey of the recolonization of lichens on trees in a large urban and suburban area has, however, been previously documented in the British Isles.

To determine what, if any, improvements of the lichen flora of the London area had taken place, 29 sites in the north and west of Greater London were studied between December 1979 and February 1980. The number of trees available for study varied but a minimum of five was examined at each site. Lichen abundance was estimated on a 1–5 scale (Table 1) and particular attention was paid to *H. physodes*, *Evernia prunastri*, *Parmelia caperata*, *Parmelia subaurifera*, *P. sulcata* and *Usnea subfloridana*, as these species have different ranges of tolerance to SO₂ (ref. 3) and are easily identified even when only a few millimetres in size. Additional interesting species were encountered in some sites, especially the Ruislip Local Nature Reserve, where 25 lichens not recorded from there in 1963 were found, including nine which are probably recent colonists (these results are presented in detail elsewhere²⁰). Other notable records include *Buellia punctata* and *Xanthoria polycarpa* on dead elms near Northwood Hills (national grid ref. TQ 104898), the former also

Fig. 1 Changes in mean annual (black) and mean winter (unshaded) SO₂ values at six recording stations (see Fig. 3) in north-west London. Based on data compiled by the Warren Spring Laboratory³⁶.

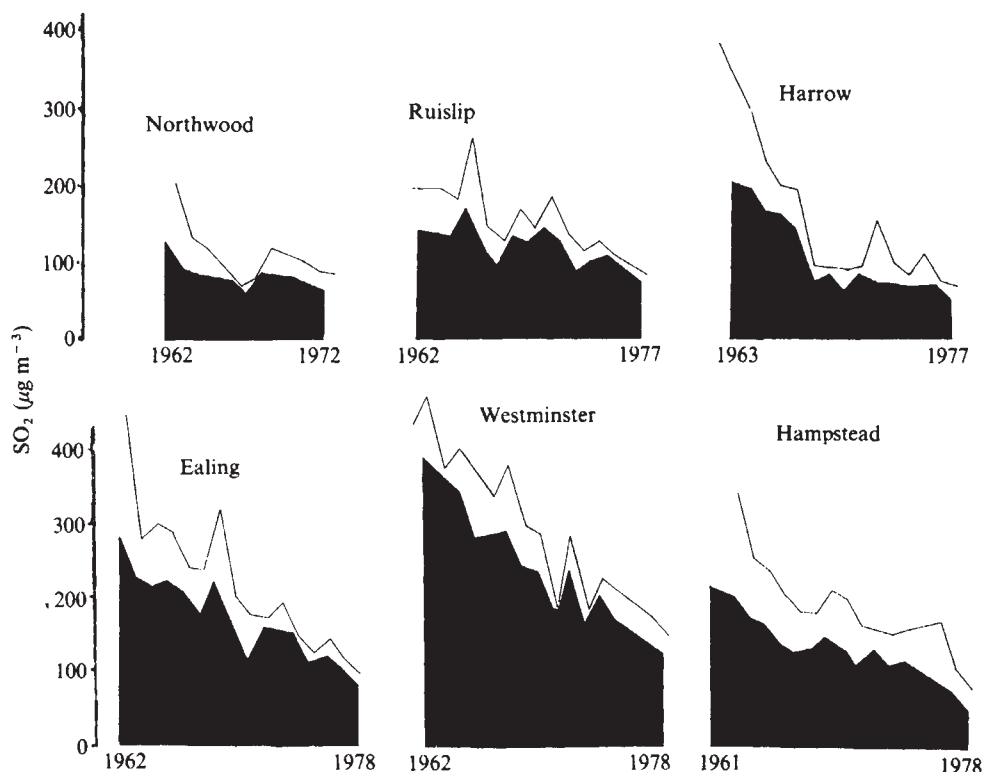


Table 1 Abundance of six lichen species at 29 sites in north-west London in 1979–80 compared with their status in Inner London in 1970†

Site	National grid ref.	<i>Evernia prunastri</i>	<i>Hypogymnia physodes</i>	<i>Parmelia caperata</i>	<i>Parmelia subaurifera</i>	<i>Parmelia sulcata</i>	<i>Usnea subfloridana</i>	Lichen zone*
Ruislip LNR†	TQ 090901	4	3	1	4	5	4	6
Ruislip Lido†	TQ 088906	3	3	—	2	4	2	5
Copse Wood†	TQ 084901	1	3	—	—	2	—	5
Park Wood†	TQ 093905	1	3	—	—	2	—	5
Brent Reservoir	TQ 217884	1	1	—	—	—	—	5
Hampstead Heath (Wildwood Road)	TQ 262886	—	2	—	—	1	—	5
Hampstead Heath (Vale of Health)	TQ 278874	1	1	—	—	2	—	5
Harrow Weald Common (south)†	TQ 139934	—	2	—	—	1	—	5
Mad Bess Wood†	TQ 075906	—	3	—	—	1	—	4
Bayhurst Wood†	TQ 068891	1	1	—	1	1	—	4
Pinner Park†	TQ 119895	—	1	—	—	—	—	4
Horsenden Hill	TQ 163854	—	1	—	—	—	—	4
Harrow Hill Pond	TQ 155870	1	1	—	1	1	—	4
Harrow Weald Common (north)†	TQ 143933	—	1	—	—	—	—	4
Ickenham Marsh†	TQ 088869	—	—	—	1	1	—	4
Hanwell Golf Course Pond	TQ 142813	—	1	—	1	1	—	4
Osterley Park	TQ 147795	—	2	—	2	2	—	4
River Thames at Kew	TQ 175777	—	—	—	—	—	—	3
River Thames at Isleworth	TQ 173777	—	—	—	—	—	—	3
River Brent at Perivale	TQ 170833	—	—	—	—	—	—	3
Lytton Fields	TQ 262891	—	—	—	—	—	—	3
Coldfall Wood	TQ 276918	—	—	—	—	—	—	3
Old Park Wood†	TQ 045926	—	—	—	—	—	—	3
West Brompton Cemetery	TQ 257778	—	—	—	—	—	—	3
River Crane at Twickenham†	TQ 133733	—	—	—	—	—	—	3
Holland Park	TQ 246802	—	—	—	—	—	—	3
Wormwood Scrubs	TQ 214823	—	—	—	—	—	—	3
Hyde Park	TQ 270800	—	—	—	—	—	—	2
Regents Park	TQ 280830	—	—	—	—	—	—	2
Status in London in 1970 (ref. 18)		Extinct; last seen ~1800	Rare or extinct; last seen 1957 at Mill Hill	Extinct; last seen ~1800	Extinct; last seen late 1800s	Rare; a single record on concrete in 1968 at Wood Green	Genus last seen ~1800	

Sites are arranged according to their rating on the Hawksworth and Rose³ scale. Most species occurred on *Quercus* and *Salix*, but records from *Betula* and *Carpinus* are also included; nutrient-rich bark is not considered.

* Zone to which the richest part of the site is referred on the 0–10 scale of Hawksworth and Rose³.

† Outside the 16-km-radius circle centred on Trafalgar Square used in the study of Laundon¹⁸.

‡ Abundance estimated on a 1–5 scale: 1, cover under 1% (1–5 thalli per tree); 2, 1–5% (5–20 thalli); 3, 5–20% (20–100 thalli); 4, 20–50% (100–1,000 thalli); 5, 50–100% (over 1,000 thalli).

in Pinner Park (TQ 135906) on dead elm stumps and *Rinodina exigua* on poplar in Eastcote (TQ 107889).

Comparison of the data presented in Table 1 with that of the same species in London in 1970 (ref. 18) leaves no doubt that there has been a marked incursion into north-west London in the past decade. To estimate the dates at which recolonization might have occurred, growth rates were studied by making direct area measurements, tracing 30 thalli of each of *H. physodes*, *P. sulcata* and *P. subaurifera* between April 1979 and March 1980 in the Ruislip Local Nature Reserve. These studies (to be reported in detail elsewhere) showed that the increases in area per unit time were significantly different (*t*-test between $P = 0.5$ and $P = 0.001$) between these three species. The annual increases in area at this site were 100–150% for *H. physodes*, 215–230% for *P. subaurifera* and 160–200% for *P. sulcata*, but no correlation between starting size and percentage increase per unit time was obtained (*H. physodes*, $R = -0.072$; *P. subaurifera*, $R = 0.019$; *P. sulcata*, $R = -0.002$; at $P = 0.05$ not significantly different from zero). In general, relative growth of circular lichen thalli expressed on a percentage basis is expected to be constant during the exponential growth phase, but to decline thereafter²¹; the exponential phase tends to last 10–15 yr and the Ruislip data therefore conform to the expected pattern. Reports of the effect of air pollutants on the growth rate of lichens are contradictory and the effect may vary both with the species and pollutant levels involved. However, a study on *Parmelia saxatilis* (a species closely related to *P. sulcata*) in north-east England indicated that growth was not significantly affected by substantial increases in ambient SO_2 levels²². Furthermore, in species where the growth rate is depressed, the

amount of depression involved would not materially affect the results reported here²³. Variations in the growth rate of a species can also arise from differences in humidity or aspect, or whether specimens are growing on vertical as opposed to horizontal surfaces^{21,24}. Based on the mid-value between the mean of

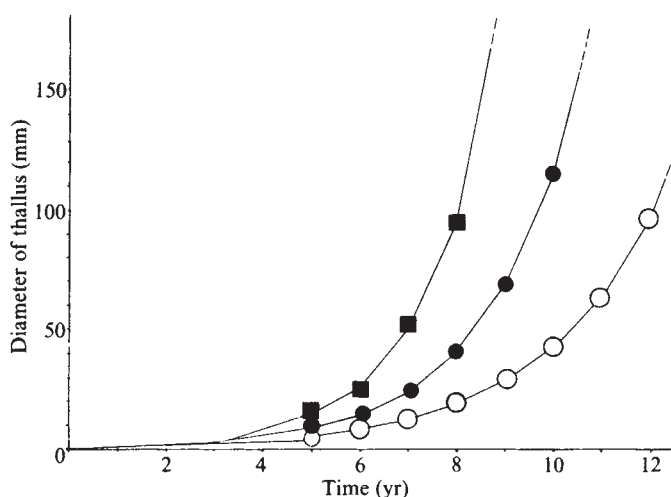


Fig. 2 Calibration curve used to estimate the minimum age of selected lichens from the diameter of their thalli. Based on the observed rates of increase in area per unit time at Ruislip Local Nature Reserve. ■, *P. subaurifera*; ●, *P. sulcata*; ○, *H. physodes*.

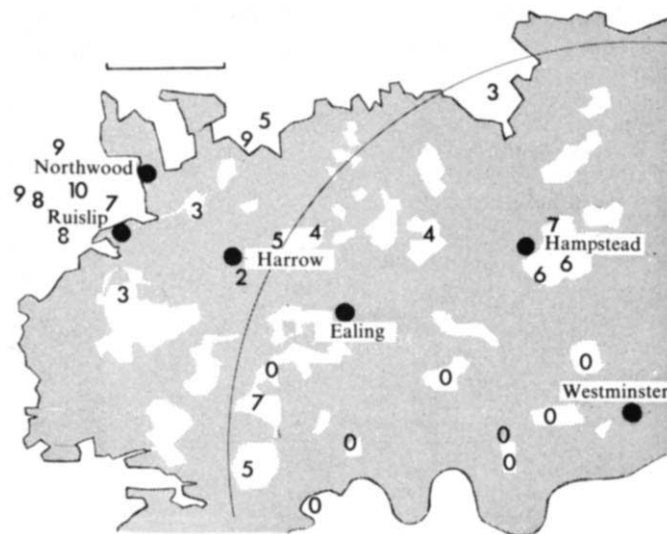


Fig. 3 North-west London showing the minimum age of foliose lichen colonies on trees (expressed in yr) calculated from observed thallus sizes and growth rates (Fig. 2). The arc indicates the limit of the survey by Laundon¹⁸ (16-km radius from Trafalgar Square). Scale bar, 5 km.

readings for each species and the median of the range of values, the expected rate of increase in area per unit time was used to construct a calibration curve relating thallus diameter to age for these species (Fig. 2). This curve is designed to place a minimum age on individual colonies of the species concerned (where many specimens are present) rather than provide unequivocal dates for each individual thallus.

The results of the growth-rate study have been incorporated into Figs 3, 4, which show that recolonization by the foliose lichens considered has, subject to the above qualifications, taken place in the past 3–7 yr at most of the sites investigated, although this process was already in operation 8–10 yr ago in the Ruislip–Northwood and Harrow Weald areas. There is some collaborative data for these latter two areas: in Harrow Weald, populations of *H. physodes* declined between 1969 and 1971 but were still present at the latter date²⁵, while in the Ruislip Local Nature Reserve, foliose lichens were well developed in 1963 (ref. 20) (unfortunately there is no information on fluctuations in this site between 1963 and 1979). The results also show that the ranges of foliose lichens in the Ruislip area have extended considerably within the last 6 yr (Fig. 4).

As there is much evidence of the sensitivity of lichens to SO_2 pollution^{3–8} and of the declines in SO_2 levels in the study area during the period when most of the lichen recolonization seems to have taken place, it is reasonable to suggest that the lichen recolonization now being seen is a direct response to falling mean SO_2 levels. Changes in levels of smoke would not be expected to cause responses in the lichen flora²⁶. Air pollutants such as nitrogen oxides and ozone, which may have increased in the London area over the past two decades, would not have any adverse effects judging from experimental studies^{27–29}. We therefore consider that these data demonstrate that London's lichens are responding to improvements in the air quality in the manner forecast by studies on deteriorating lichen floras. They also demonstrate that a cover of *L. conizaeoides* is insignificant in preventing the establishment of foliose lichens; indeed *H. physodes* and *P. sulcata* can establish directly on *L. conizaeoides*. Further, the lichenicolous fungus *Athelia arachnoidea*, which can kill many lichens and is especially common on *L. conizaeoides* in suburban areas, does not seem to oppose significantly re-establishment, as has been suggested³⁰. It is also of interest that the sites in which recent recolonization has been found are not all contiguous. Recolonization has been more rapid in areas already with a nearby source of appropriate propagules, but dispersal has evidently been possible over

considerable distances. Lichen diaspores are widespread in the air spora but soredia can also be carried on the feet of birds; as demonstrated at Brent Reservoirs¹⁹, a site included in this survey.

The most suitable sites for recolonization are ones with well lit sheltered trees, in humid sites (for example, Ruislip Local Nature Reserve, Harrow Hill Pond, Wildwood Road), and often with willows overhanging a pond or marshy area surrounded by dense shrubs. In woodland sites, the earliest colonization tends to be at ground level, where air movements are less and the microclimate more favourable to growth; this would be expected as foliose lichens remain longest on tree bases in conditions of increasing SO_2 (ref. 3). In larger woodlands where the tree bases are very shaded and lichen growth is precluded, lichen communities including foliose species tend to develop on horizontal branches higher in the canopy where they can be easily missed. On willow, the first foliose lichen to colonize is *H. physodes*, followed by *P. subaurifera* and *P. sulcata*; *P. subaurifera* then tends to become more common (frequently growing where other species have died off) and *P. sulcata* dominant. On dry oak or birch, *H. physodes* seems better able to withstand competition from other species.

The investigations reported here concern only trees and studies in selected sites. We have not been able to undertake a tree-by-tree survey throughout north-west London and some recolonization sites will almost certainly have been overlooked. The sites listed in Table 1 are consequently representative rather than definitive as to the present position. Although the present survey was restricted to north-west London, the current improvement may be more general. For example, specimens of *Hypogymnia physodes* to 5 mm long were discovered on an oak stump near Pembroke Lodge in Richmond Park by F. S. Dobson in November 1980; this specimen was last recorded here in 1929 (ref. 17).

Lichens on man-made substrates, such as brick and asbestos-cement, can also be used as indicators of air pollutants but relatively little information is available on the way in which they respond, which is complicated by variations in the chemical nature of the substrate. However, our superficial observations suggest that they are responding in a parallel manner to the results presented here. In particular, *Lecanora muralis*, a species which changes its substrate range as SO_2 levels increase²³, can now grow on less basic substrates in the Kew area than it did in 1970; increases in this species have even led to comments in the local press arising from the general public's concern about this 'green fungus' on pavements^{31,32}.

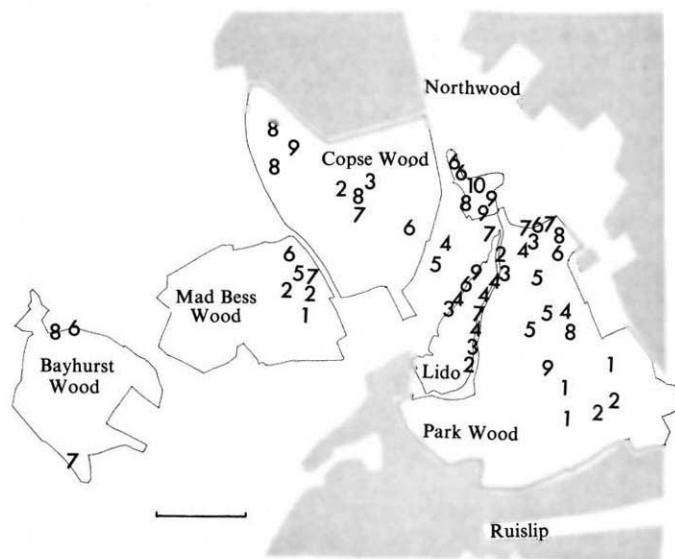


Fig. 4 Ruislip–Northwood Woods showing the minimum age of foliose lichen colonies on trees (expressed in yr) calculated from observed thallus sizes and growth rates (Fig. 2). Scale bar, 500 m.

The lichen flora of London has been studied since the early seventeenth century³³ and the historical records were combined into the survey of Laundon^{18,34}. These records show a serious and progressive decline starting at least by the early nineteenth century, but which was particularly pronounced in the later decades of that century (for example, in Epping Forest²⁵) and continued into the 1950s–60s. The loss of 129 lichen species from the area within a 16-km radius of Trafalgar Square has been attributed to increases in air pollution between 1800 and 1970 (ref. 18). Although the slight improvements reported here are encouraging, more drastic reductions in mean SO₂ levels are required before the environment is again suitable for many of the eliminated species. Indeed, a reduction in Inner London

from the current mean of about 130 µg m⁻³ to around 40–50 µg m⁻³ would be necessary for survival of many of the more attractive foliose and fruticose species; transplant experiments have shown how hostile the Inner London environment is to some of these³⁵. It has been estimated that mean SO₂ levels in urban areas will tend to fall until 1985, but thereafter return to 1975 levels due to changes in fuel-mix¹. If this is true, only a limited rather than a substantial improvement in the lichen flora of the area seems likely.

We thank Mr P. W. James, Mr M. P. Jones, Mr J. R. Laundon and Drs C. J. Muskett and F. Rose for help. This work was carried out while C.I.R. was in receipt of a Chelsea College (University of London) Research Studentship.

Received 2 September; accepted 12 November 1980.

1. The United Kingdom Environment 1979: Progress of Pollution Control, Pollut. Pap. no. 16 (HMSO, London, 1979).
2. Sanford, H. *Lond. Nat.* **58**, 89–92 (1979).
3. Hawksworth, D. L. & Rose, F. *Nature* **227**, 145–148 (1970).
4. Ferry, B. W., Baddeley, M. S. & Hawksworth, D. L. (eds) *Air Pollution and Lichens* (Athlone Press of the University of London, London, 1973).
5. Hawksworth, D. L. *Lichenologist* **6**, 122–125 (1974); **7**, 62–66, 173–177 (1975); **8**, 87–91, 179–182 (1976); **9**, 77–82, 147–151 (1977); **10**, 95–100 (1978).
6. Hawksworth, D. L. & Henderson, A. *Lichenologist* **10**, 227–230 (1978); Henderson, A. & Hawksworth, D. L. *Lichenologist* **11**, 91–95 (1979).
7. Henderson, A. *Lichenologist* **11**, 313–319 (1979); **12**, 145–148, 397–402 (1980).
8. Hawksworth, D. L. & Rose, F. *Lichens as Pollution Monitors*, Stud. Biol. no. 66 (Arnold, London, 1976).
9. Jüring, P. *Bibliotheca lich.*, *Lehre* **4**, 1–164 (1975).
10. Skye, E. & Hallberg, I. *Oikos* **20**, 547–552 (1969).
11. Westman, L. *Wahlenbergia* **2**, 1–146 (1975).
12. Lindsay, D. C. *Proc. Bgham nat. Hist. Soc.* **23**, 234–249 (1978).
13. Seaward, M. R. D. *Proc. Leeds lit. phil. Soc.*, *Sci.* **10**, 141–208 (1976).
14. Henderson, A. *Naturalist, Hull* **1977**, 141–144 (1977).
15. Henderson-Sellers, A. & Seaward, M. R. D. *Environ. Pollut.* **19**, 207–213 (1979).
16. Gilbert, O. L. *Lichenologist* **12**, 325–395 (1980).
17. Hawksworth, D. L. *Lichenologist* **4**, 105–193 (1969).
18. Laundon, J. R. *Lond. Nat.* **49**, 20–69 (1970).

19. Bailey, R. H. & James, P. W. *Lichenologist* **11**, 105–106 (1979).
20. Hawksworth, D. L. & Rose, C. I. *J. Ruislip Distr. nat. Hist. Soc.* **22**, 23–29 (1979).
21. Topham, P. B. in *Lichen Ecology* (ed. Seaward, M. R. D.) 31–68 (Academic, London, 1977).
22. Gilbert, O. L. *Lichenologist* **5**, 11–17 (1971).
23. Seaward, M. R. D. in *Lichenology: Progress and Problems* (eds Brown, D. H., Hawksworth, D. L. & Bailey, R. H.) 323–357 (Academic, London, 1976).
24. Armstrong, R. A. thesis, Univ. Oxford (1974).
25. Hawksworth, D. L., Rose, F. & Coppins, B. J. in *Air Pollution and Lichens* (eds Ferry, B. W., Baddeley, M. S. & Hawksworth, D. L.) 330–367 (Athlone Press of the University of London, London, 1973).
26. Hawksworth, D. L. in *Air Pollution and Lichens* (eds Ferry, B. W., Baddeley, M. S. & Hawksworth, D. L.) 38–76 (Athlone Press of the University of London, London, 1973).
27. Brown, D. H. & Smirnoff, N. *Lichenologist* **10**, 91–94 (1978).
28. Nash, T. & Sigal, L. *Bryologist* **82**, 280–285 (1979).
29. Nash, T. *Bryologist* **79**, 103–106 (1976).
30. Arvidsson, L. *Svensk bot. Tidskr.* **72**, 285–292 (1979).
31. *Brentford & Chiswick Times*, 11 January (1980).
32. Gilbert, J. L. *Bull. Br. Lichen Soc.* **46**, 7 (1980).
33. Hawksworth, D. L. & Seaward, M. R. D. *Lichenology in the British Isles 1568–1975* (Richmond Publishing, Richmond, 1977).
34. Laundon, J. R. *Lichenologist* **3**, 277–327 (1967).
35. Ferry, B. W. & Coppins, B. J. *Lichenologist* **11**, 63–77 (1979).
36. Warren Spring Laboratory *The Investigation of Air Pollution. National Survey. Smoke and Sulphur Dioxide* (Department of Trade and Industry, Stevenage, 1962–80).

Vacuoles as storage compartments for nitrate in barley leaves

Enrico Martinoia, Urs Heck & Andres Wiemken

Department of General Botany, Swiss Federal Institute of Technology, Sonneggstrasse 5, CH-8092 Zürich, Switzerland

Nitrate, the principal nitrogen source of most plants, can accumulate in large quantities in certain crop plants, notably members of the Chenopodiaceae (spinach and beet), Gramineae, Cruciferae (radish and kale) and Compositae (lettuce). Concentrations may exceed 2% fresh weight (17–24% dry weight) in extreme physiological conditions¹. This is alarming because nitrate is readily reduced in organisms to the toxic nitrite, which may react with amines to form very potent carcinogenic nitrosamines². Large stores of nitrate can be maintained in plant cells even in the presence of high nitrate reductase activity^{3–8}. Incoming nitrate does not seem to mix with existing stores^{4–6,9} and a steady influx of nitrate into the cells was found to be necessary to keep nitrate reductase stably induced^{3,6}. Such observations have been explained by postulating a small 'metabolic' pool of nitrate accessible to nitrate reductase⁴ and presumably also responsible for the induction of the enzyme³, and a large 'storage' pool separate from the sites of metabolism^{3–9,14}. Repeated attempts to measure these pools by an indirect method⁴ have given equivocal results^{1,10}, but storage pools of nitrate have been generally thought to be located in vacuoles, which are difficult to isolate and analyse. However, we were able to isolate and purify the large central vacuoles of barley mesophyll cells and found most of the accumulated nitrate in the vacuoles.

Vacuoles were isolated from protoplasts as described in Fig. 1 legend and purity was confirmed by phase contrast microscopy (Fig. 1c). Contamination with intact protoplasts never exceeded 5%. Purity was further tested using marker enzymes. The

specific activity of α -mannosidase^{11,12} was much higher in the vacuolar fractions than in the homogenate of whole protoplasts (Table 1). Activities of α -mannosidase measured per 10⁶ protoplasts and per 10⁶ vacuoles were about the same (Table 1), indicating that almost all the enzyme was located in the vacuoles, of which each protoplast has only one (Fig. 1b). The vacuolar fraction contained 13% of the total activity of α -mannosidase present in the homogenate of protoplasts. On a basis of number protoplasts lysed the yield of α -mannosidase (that is of vacuoles) was 26%. The activities of two other hydrolytic enzymes, β -N-acetylglucosaminidase and protease, were also mostly restricted to the vacuoles (Table 2), supporting results obtained with other plants^{11–13,15}. However, enzymes attributed to the chloroplasts (aldolase) and the cytosol (glucose-6-P isomerase, nitrate reductase¹⁶) were hardly detectable in the vacuolar preparation (Table 2).

We measured the contents of nitrate and amino acids in our pure preparation of vacuoles. As soluble substances can be easily lost from the vacuoles by leakage during isolation, we were surprised to find that almost all the nitrate in the protoplasts was contained in the vacuoles, together with about half of the soluble amino acids (Table 2). Vacuolar pools of amino acids have been reported elsewhere^{17–19}.

This demonstration of the vacuolar localization of nitrate raises the question of the value of nitrate storage pools to the plants. These pools could be nitrogen reserves. The rapid accumulation of nitrate from the soil could also help to prevent

Table 1 α -Mannosidase as vacuolar marker

	α -Mannosidase activity (nmol min ⁻¹)	
	Protoplasts	Vacuoles
Per (mg) protein	6.8	138*
Per 10 ⁶ protoplasts or vacuoles	8.6	8.3†

* The specific activity of α -mannosidase in the isolated vacuoles is 20.3 times higher than that in the homogenate of protoplasts.

† Assuming that each protoplast has only one vacuole, 97% ($\pm$ 12%) α -mannosidase was found in the vacuoles. Procedures described in Table 2 legend.

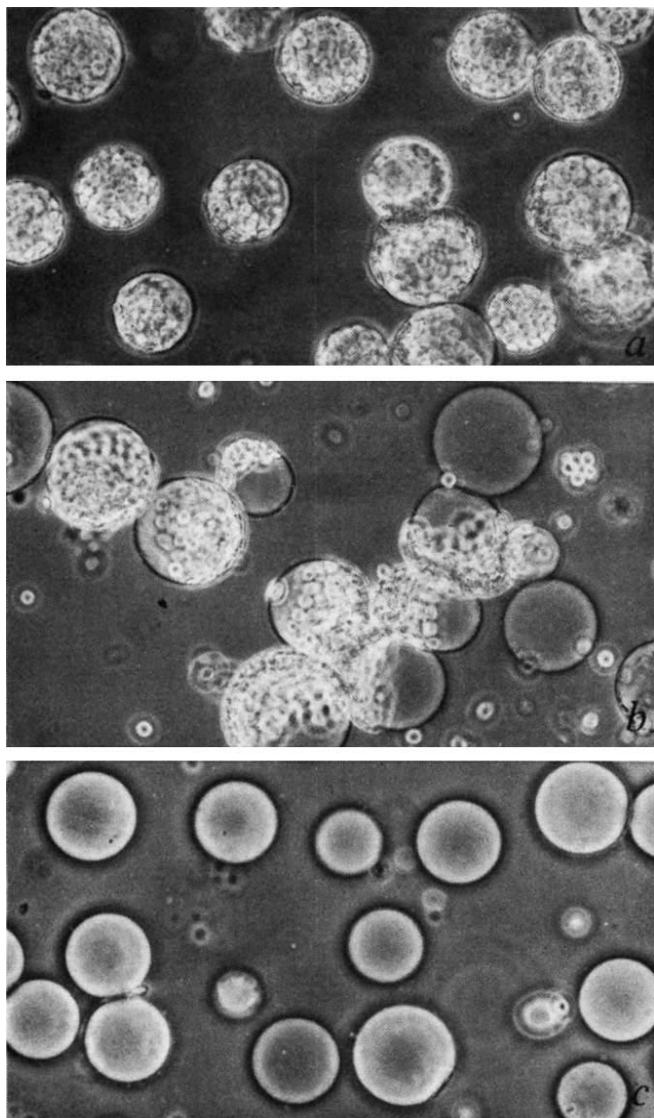


Fig. 1 *a*, Barley mesophyll protoplasts; *b*, protoplasts releasing their single large vacuole; *c*, isolated vacuoles. Phase contrast micrographs $\times 300$. Protoplasts were prepared from the mesophyll of primary leaves of barley seedlings (*Hordeum vulgare* L. cv Nympe) grown for 8 days on vermiculite, irrigated with 30mM KNO_3 (27 °C, 12 h light/dark cycle, 60% humidity). The leaves were cut into pieces (1 mm²) which were incubated in medium containing 2% Onozuka R-10 cellulase (supplied by Welling & Co. Hamburg, Germany), 1% Pectolyase (Seishin Pharma, Noda, Chiba, Japan), 0.4M sucrose, 20mM citrate-Na⁺ buffer, pH 5.6 (2 g fresh weight of leaves per 10 ml in a Petri dish). After 3 h at 20 °C the protoplasts, which came only from the mesophyll, were filtered through a 80 μm nylon net and purified by flotation (7 min at 1,400g in a swingout rotor) through a three-step density gradient: (step 1), 10 ml suspension of protoplasts diluted with 10 ml of 10% Ficoll 400 (Pharmacia Uppsala, Sweden), 0.4M sucrose, and 'supplements' (15mM phosphate buffer (pH 7.6), 2mM EDTA-Na (but not for nitrate determination), 10mM cysteine-HCl (only in nitrate reductase determination)); (step 2), 10 ml 2.5% Ficoll, 0.4M sucrose and 'supplements'; (step 3), 4 ml 0.4M sorbitol and 'supplements'. The protoplasts recovered after centrifugation from the interface above step 2 were diluted with an equal volume of 10% Ficoll, 0.4M sucrose plus 'supplements' and floated a second time through an identical density gradient. Vacuoles were liberated from the purified protoplasts by pressing the suspension (3×10^6 protoplasts per ml, 4 °C) through a long needle (10 cm $\times$ 0.2 mm) using a syringe. After this treatment about half of the protoplasts were ruptured and about two thirds of these released their single large vacuole intact. The vacuoles were immediately purified by flotation (2 min at 200g, followed by 3 min at 1,000g in a swingout rotor) through a three-step density gradient: (step 1), 5 ml lysate in 0.4M sucrose, 2.5% Ficoll and 'supplements'; (step 2), 5 ml 0.2M sucrose, 0.2M sorbitol and 'supplements'; (step 3), 2 ml 0.4M sorbitol and 'supplements'. Purified vacuoles were recovered from the step 3 layer. The whole isolation procedure from the time of rupturing of the protoplasts, was no longer than 8 min.

Table 2 Subcellular localization of enzymes and substances

	Per 10 ⁶ protoplasts	Activities or contents Per 10 ⁶ vacuoles	% In vacuoles
Vacuolar marker enzymes:			
α -Mannosidase*	8.6	8.3	100
β -N-Acetylglucosaminidase*	12.8	11.0	89
Protease†	5.1	4.2	85
Extravacuolar marker enzymes:			
Aldolase*	82	0.3	<1
Malate dehydrogenase*	489	15.0	3
Glucose-P isomerase*	130	6.4	5
Nitrate reductase‡	40	<0.5	<1
Protein§	1.27	0.06	4.7
Amino acids	1,165	585	52
Nitrate	1,460	1402	99

Protoplasts were counted in a haemocytometer. The number of vacuoles was calculated from the α -mannosidase activity (the activity of α -mannosidase per 10⁶ vacuoles (Table 1) was known and remained fairly constant ($\pm 8\%$) from one experiment to another). Per cent in vacuoles was calculated by assuming that 100% of the α -mannosidase is located in the vacuoles. The calculation was as follows: % activity or content in the vacuolar fraction divided by % activity of α -mannosidase in the same fraction multiplied by 100. Enzyme activities were measured by standard methods: aldolase²², malate dehydrogenase²², nitrate reductase²³, α -mannosidase and *N*-acetylglucosaminidase with the corresponding nitrophenol substrates¹², and acid protease with haemoglobin as substrate in 0.1M sodium acetate buffer, pH 3.9, as described elsewhere¹¹. Amino acids were measured with ninhydrin and protein by a modified method of Lowry¹¹. Nitrate concentrations were determined potentiometrically with an ion-selective liquid membrane electrode as described elsewhere^{24,25}. The membrane composition was 6% (by weight) methyl-tridodecyl-ammonium-chloride, 65% dibutylphthalate and 29% polyvinylchloride (by weight). The calibration for measurements in solution with protoplast lysate were done in solutions of the composition given in Fig. 1 legend (step 2) and in 10^{-4} , 10^{-3} and 10^{-2} M sodium nitrate. For the determination of nitrate in vacuoles the solution of step 3 was used as background. The detection limit for this experiment was checked using barley which had not been irrigated with nitrate.

* nmol min⁻¹.

† μg bovine serum albumin (BSA) equivalents per min.

‡ nmol NO₂⁻ h⁻¹.

§ μg BSA equivalents.

|| nmol.

leakage, or uptake by neighbouring plants. It may also play a part in ionic and osmotic balance of the cells. This is particularly likely in plants in which nitrate reaches high concentrations of up to 300 mM (ref. 1) (generally halophytes) and in which nitrate may be accumulated as an alternative to chloride²⁰. The answer to the question on the control of transport of nitrate through the vacuolar membrane could shed light on the relationship between the nitrate pools and the highly complex regulation of nitrate reductase activity²¹.

We thank D. Ammann and P. Anker for help with nitrate measurements and S. Türlér for reading, and D. Furrer for typing the manuscript.

Received 1 September; accepted 6 November 1980.

- Hewitt, E. J. Huckleby, D. P., Mann, A. F., Notton, B. A. & Rucklidge, G. J. in *Nitrogen Assimilation of Plants* (eds Hewitt, E. J. & Cutting, C. V.) 255-287 (Academic, London, 1979).
- Walters, C. L. & Walker, R. in *Nitrogen Assimilation of Plants* (eds Hewitt, E. J. & Cutting, C. V.) 637-648 (Academic, London, 1979).
- Heimer, J. & Filner, P. *Biochim. biophys. Acta* **230**, 362-372 (1971).
- Ferrari, E. T., Yoder, O. C. & Filner, P. *Pl. Physiol.* **51**, 423-431 (1973).
- Aslam, M., Oaks, A. & Huffaker, R. C. *Pl. Physiol.* **58**, 588-591 (1976).
- Shaner, D. L. & Boyer, J. S. *Pl. Physiol.* **58**, 499-504 (1976).
- Jones, R. W. & Sheard, R. W. in *Nitrogen Assimilation of Plants* (eds Hewitt, E. J. & Cutting, C. V.) 521-539 (Academic, London, 1979).
- Ashley, D. A., Jackson, W. A. & Volk, R. J. *Pl. Physiol.* **55**, 1102-1106 (1975).
- Martin, P. Z. *Pflanzenphysiol.* **70**, 158-165 (1973).
- Hageman, R. H., Reed, A. J., Femmer, R. A., Sherrard, J. H. & Dalling, M. J. *Pl. Physiol.* **65**, 27-32 (1980).
- Wiemken, A., Schellenberg, M. & Urech, L. *Archs Microbiol.* **123**, 23-35 (1979).
- Boller, Th. & Kende, H. *Pl. Physiol.* **63**, 1123-1132 (1979).
- Nishimura, M. & Beevers, H. *Pl. Physiol.* **62**, 44-48 (1978).
- Oaks, A. & Bidwell, R. G. S. *An. Rev. Pl. Physiol.* **21**, 43-66 (1970).
- Matile, P. *An. Rev. Pl. Physiol.* **29**, 193-213 (1978).
- Wallsgrove, R. M., Lea, P. J. & Milfin, B. J. *Pl. Physiol.* **63**, 232-236 (1979).
- Wiemken, A. & Dürr, M. *Archs Microbiol.* **101**, 45-57 (1974).
- Dürr, M., Urech, K., Boller, Th., Wiemken, A., Schwencke, J. & Nagy, M. *Archs Microbiol.* **121**, 169-175 (1979).

19. Sasse, F., Backs-Hüsemann, D. & Barz, W. *Z. Naturf.* **34c**, 848–853 (1979).
20. Stewart, G. R. in *Nitrogen Assimilation of Plants* (eds Hewitt, E. J. & Cutting, C. V.) 651–652 (Academic, London, 1979).
21. Srivastava, H. S. *Phytochem.* **19**, 725–733 (1980).
22. Bergmeyer, H. U. *Methods of Enzymatic Analysis* 2nd edn (Verlag and Academic, New York and London, 1974).
23. Hageman, R. H. & Huckleby, D. P. *Meth. Enzym.* **23**, 491–503 (1971).
24. Hartman, K. *et al. Mikrophim. Acta* **11**, 235–246 (1978).
25. Nielsen, H. J. & Hansen, E. H. *Analytica chim. Acta* **85**, 1–16 (1976).

Subpopulations of cultured chick sympathetic neurones differ in their requirements for survival factors

David Edgar, Yves-Alain Barde & Hans Thoenen

Abteilung Neurochemie, Max-Planck-Institut für Psychiatrie, D-8033 Martinsried, FRG

Peripheral autonomic and sensory neurones derived from the neural crest will survive *in vitro* only if the culture medium is supplemented with specific factors (for review see ref. 1). For example, the nerve growth factor (NGF), although supporting the survival of sympathetic and spinal sensory neurones², is ineffective on parasympathetic neurones³, whereas medium conditioned by chick heart cells (HCM) supports the survival of all three neuronal types⁴. We showed previously that the requirements of cultured spinal sensory ganglion neurones for survival factors changed during development, and so provided a basis for the classification of such factors⁵. We now demonstrate that cultured post-mitotic neurones from chick paravertebral sympathetic ganglia respond differentially to NGF, HCM and medium conditioned by C6 glioma cells (GCM). Thus, these three survival factors are functionally distinct in that they support the survival in culture of discrete subpopulations of sympathetic neurones. Those subpopulations responding to HCM and NGF are shown to differ not only in their requirements for survival factors but also in their contents of the cholinergic and adrenergic marker enzymes choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH).

Cultures were established using methods similar to those previously described^{5,6}, the age of the chick embryos being checked by staging according to the criteria of Hamburger and Hamilton⁷. Lumbar and sacral paravertebral sympathetic ganglia were taken from embryos incubated between 8 and 18 days, dissociated and the cells pre-plated to remove most of the non-neuronal cells^{5,6}. The few remaining non-neuronal cells in the cultures were easily distinguishable from neurones, the latter having compact, round cell bodies with thin neurite processes at least five cell diameters in length. NGF, GCM and anti-NGF antibodies were prepared as previously described, GCM being used in the present experiments after 10-fold concentration⁵. HCM was prepared by the method of Collins⁸, except that 5% heat-inactivated horse serum was substituted for fetal calf serum to cultivate the heart cells, and the HCM was concentrated two-fold by ultrafiltration (Amicon UM 10) before use.

After removal of most of the non-neuronal cells by pre-plating and in the absence of added factors, less than 0.1% of the sympathetic neurones taken from 12-day old embryos survived after 2 days in culture (Table 1). Addition of NGF, HCM or GCM at the start of the culture period resulted in the survival of differing proportions of neurones, although each of the three factors was shown to be at saturating concentration: doubling the concentration of a given factor did not increase the number of surviving neurones. Culture with a combination of factors, however, resulted in an additive effect (Table 1), a maximum of 90% 12-day old neurones surviving in the presence of both NGF and HCM. Because such a high survival is possible in culture,

Table 1 Survival of cultured 12-day chick sympathetic neurones

% Neuronal survival in response to:				
Factor shown	Double concentration of factor	Factor plus NGF (5 ng ml ⁻¹)	Factor plus HCM	
Control medium	0.09 ± 0.10			
NGF (5 ng ml ⁻¹)	37.1 ± 4.5	39.0 ± 5.0		
HCM	45.0 ± 3.0	41.0 ± 6.0	88 ± 9.5	
GCM	6.1 ± 2.1	8.0 ± 2.5	55 ± 4.5	71 ± 7.8

Mean values ± s.e.m. are given ($n = 3$). Sympathetic neurones were taken from paravertebral ganglia of 12-day old embryonic chicks. After trypsinization and dissociation, the cells were pre-plated in 10-cm plastic tissue culture dishes for 150 min as previously described^{5,6}. The culture medium was F14 (ref. 14), supplemented with 10% heat-inactivated horse serum, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). The neuronal suspension was then removed, those cells excluding trypan blue (>90%) counted using a haemocytometer and plated in poly-DL-ornithine-coated dishes⁸ at a density of 5,000–8,000 cells per 35-mm dish (equivalent to one ganglion) in 1.5 ml medium containing the factors shown. Where NGF was not added, the media were supplemented with 500 ng ml⁻¹ anti-NGF antibodies to inhibit any endogenous NGF, as found, for example, in GCM⁵. Neurones were counted after 48 h culture, the number being checked to remain constant on two subsequent days.

Table 2 Enzyme levels of 12-day chick sympathetic neurones

	Specific activity (pmol per min per mg) ± s.e.m. ($n = 3$)		Ratio ChAT/TH
	ChAT	TH	
After 48 h culture with:			
NGF	102 ± 44	281 ± 75	0.36
HCM	842 ± 77	137 ± 31	6.07
NGF + HCM	855 ± 45	310 ± 40	2.76
After 48 h culture with NGF, followed by 48 h with both NGF and HCM	85 ± 30	210 ± 40	0.40
Paravertebral sympathetic chains	680 ± 28	266 ± 20	2.56

Cultures were set up as described in Table 1 legend, the number of cultured neurones being increased to 50,000–80,000. After culture for 2 days, neurones were collected and ChAT activity was measured¹⁵ using the ChAT inhibitor naphthylvinyl pyridine¹⁶ (50 µM) to confirm specificity of the assay. Tyrosine hydroxylase was measured by a modification of the method of Levitt *et al.*¹⁷, the difference being that a tetrahydrobiopterin cofactor (640 µM) was used¹⁸, in the presence of 20 mM ascorbate, 10⁻⁴ M ferrous ammonium sulphate and 24,000 U ml⁻¹ catalase¹⁹. The tyrosine concentration was 23 µM and specificity of the assay was checked using the TH inhibitor α -methyl-tyrosine (10⁻⁴ M)²⁰. Initial reaction rates were linear with time and amount of sample. Protein was estimated by the method of Bradford²¹ using bovine γ -globulins as the standard.

and because there was no great loss of cells during the dissociation procedure which led to a complete disruption of the ganglia, this system provides an opportunity to observe *in vitro* a representative picture of the whole neurone population present *in vivo*. The differing proportions of neurones surviving in response to the different factors and the additivity of the effects of those factors point to the presence of discrete subpopulations of sympathetic neurones, the survival in culture of each being selectively supported by a given factor. This conclusion supports and extends the previous observation that not all chick sympathetic neurones respond to NGF in cultured ganglion explants⁹.

To analyse further the relationships between the survival factors and their responsive neurones, we examined the effects of NGF, HCM and GCM on the survival of neurones taken from sympathetic ganglia of different embryonic ages. Figure 1 shows

that NGF supports the survival of a maximum of 37% cultured neurones taken from 12-day old embryos, whereas HCM is maximally active on 14-day old neurones (57% survival) and the effect of GCM increases with age throughout the time period studied. The developmental patterns of sympathetic neuronal survival in response to NGF and GCM are strikingly similar to those previously obtained for dorsal root ganglion neurones⁵. Thus, NGF and GCM act as functionally distinct factors for both sensory and sympathetic neurones, and HCM may be differentiated from the other two on the basis of the age of the neurones on which it is maximally active. We have not demonstrated whether those neurones which are maximally dependent on NGF for their survival at day 12 become dependent on either HCM or GCM later during development. Furthermore, a relationship remains to be established between the various subpopulations defined here by their factor requirements in culture, and those subpopulations defined by morphological criteria *in situ*¹⁰.

To approach these problems, we characterized biochemically the two major subpopulations of 12-day old neurones which survived in culture in response to either HCM or NGF. Table 2 shows that the HCM- and NGF-responsive neurones differ in their contents of TH and ChAT, necessary for the synthesis of the sympathetic neurotransmitters noradrenaline and acetylcholine, respectively. Thus, the cultured HCM-responsive neurones have a ChAT/TH ratio of 6.1, some 17-fold greater than that of those neurones surviving with NGF. Cells cultured with both NGF and HCM have an intermediate ChAT/TH ratio of 2.8, similar to that determined for freshly dissected 12-day old sympathetic chains.

We do not know whether the high ChAT levels found in the HCM-responsive neurones is a consequence of a selection of those cells in culture which had pre-existing high ChAT activity *in vivo* or whether this enzyme was induced in the chick neurones by HCM in a manner similar to that shown for neonatal rat sympathetic neurones (for review see ref. 11). Note in this connection that HCM maintains the ChAT activity in cultured neurones at the levels measured in the embryonic sympathetic chains (Table 2, see also ref. 12) at a time when preganglionic (cholinergic) innervation is largely undeveloped¹³, that is, when most of the ChAT may be in ganglionic neurones rather than innervating axons. Furthermore, HCM does not induce ChAT activity in that subpopulation of neurones previously selected by culture with NGF (Table 2). These observations are consistent with the hypothesis that NGF and HCM selectively support the survival of distinct subpopulations of embryonic chick sympathetic neurones which differ in their contents of adrenergic and cholinergic marker enzymes.

We thank Veronika Böhm for technical assistance.

Received 26 August; accepted 19 November 1980.

1. Varon, S. S. & Bunge, R. P. A. *Rev. Neurosci.* **1**, 327-361 (1978).
2. Levi-Montalcini, R. & Angeletti, P. U. *Dev. Biol.* **7**, 653-659 (1963).
3. Helfand, S. L., Smith, G. A. & Wessels, N. K. *Dev. Biol.* **50**, 541-547 (1976).
4. Helfand, S. L., Riopelle, R. T. & Wessels, N. K. *Exp. Cell Res.* **113**, 39-45 (1978).
5. Barde, Y. A., Edgar, D. & Thoenen, H. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1199-1203 (1980).
6. Greene, L. A. *Dev. Biol.* **58**, 96-105 (1977).
7. Hamburger, V. & Hamilton, H. L. *J. Morph.* **88**, 49-92 (1951).
8. Collins, F. *Dev. Biol.* **65**, 50-57 (1978).
9. Chamley, J. H., Mark, G. E., Campbell, G. R. & Burnstock, G. *Z. Zellforsch.* **135**, 287-314 (1972).
10. Edds, L. L. & van Horn, C. *J. comp. Neurol.* **191**, 65-76 (1980).
11. Patterson, P. H. A. *Rev. Neurosci.* **1**, 1-17 (1978).
12. Marchisio, P. C. & Consolo, S. *J. Neurochem.* **15**, 759-764 (1968).
13. Ross, L. L., Cosio, L., Witczak, L. & Smolen, A. *Anat. Rec.* **190**, 525 (1978).
14. Vogel, Z., Sytkowski, A. J. & Nirenberg, M. W. *Proc. natn. Acad. Sci. U.S.A.* **69**, 3180-3184 (1972).
15. Fonnum, F. *J. Neurochem.* **24**, 407-409 (1975).
16. White, H. L. & Cavallito, C. J. *J. Neurochem.* **17**, 1579-1589 (1970).
17. Levitt, M., Gibb, J. W., Daly, J. W., Lipton, M. & Udenfriend, S. *Biochem. Pharmac.* **16**, 1313-1321 (1967).
18. Black, I. B. *Brain Res.* **95**, 170-176 (1975).
19. Lerner, P., Nöse, P., Ames, M. P. & Lovenberg, W. *Neurochem. Res.* **3**, 641-651 (1978).
20. Udenfriend, S., Nirenberg, P. Z. & Nagatsu, T. *Biochem. Pharmac.* **14**, 837-845 (1965).
21. Bradford, M. M. *Analyt. Biochem.* **72**, 248-254 (1976).

The number of polarizing region cells required to specify additional digits in the developing chick wing

C. Tickle

Department of Biology as Applied to Medicine, The Middlesex Hospital Medical School, Cleveland Street, London W1P 6DB, UK

A group of mesenchyme cells at the posterior margin of the developing wing—the polarizing region—can have a dramatic effect on the pattern of structures which develop across the antero-posterior axis of the limb. If one grafts a polarizing region to the anterior margin of a wing bud so that this bud now has one polarizing region at the anterior margin and one at the posterior margin, the wing that develops has duplicated structures across the antero-posterior axis in mirror-image symmetry¹⁻³. That is, the normal pattern of digits 2 3 4 (reading from anterior to posterior) becomes 4 3 2 2 3 4. The ability of the polarizing region to specify additional digits from adjacent tissue can be progressively attenuated by γ -ray radiation⁴. If this attenuation is caused by progressively fewer cells remaining viable to signal, there should be a quantitative relationship between the number of polarizing cells used and the digit specified next to the graft. Here, two tests are reported which confirm this idea. The results suggest that the apical ridge cooperates with a small number of polarizing region cells in a monolayer to specify structures across the antero-posterior axis of the wing.

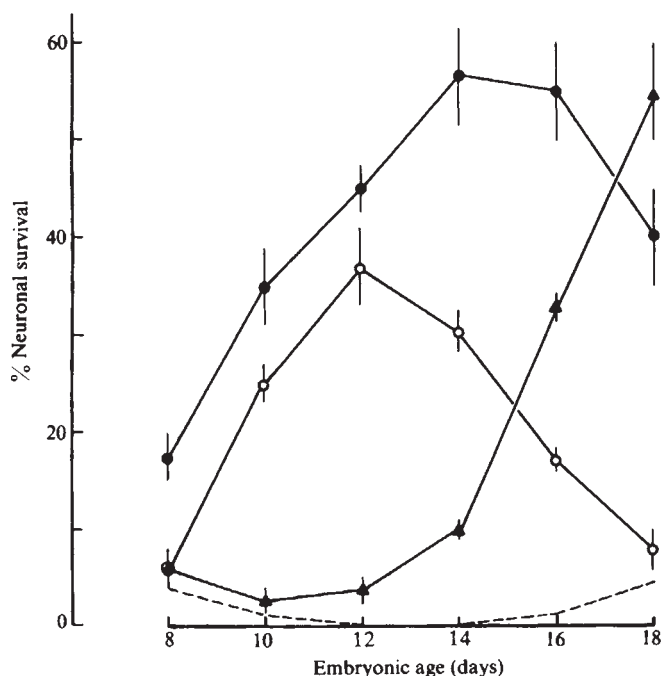


Fig. 1 Neurones were dissociated from paravertebral sympathetic ganglia taken from chicks of embryonic ages shown. After pre-plating, 5,000–8,000 neurones were plated on poly-DL-ornithine as described in Table 1 legend. Neuronal survival was estimated between days 2 and 5 after the start of culture, and it was checked that the percentage neuronal survival did not decrease significantly on two subsequent days. The dashed line shows the low basal levels of neuronal survival without added factors. An increased neuronal survival seen in control cultures of neurones from 8- and 18-day old chicks is a consequence of the slower death rate of the younger cells, and of the presence of more non-neuronal cells which support neurones in the cultures from older embryos. Parallel cultures were set up with supplements of: 5 ng ml⁻¹ NGF (○), HCM plus 500 ng ml⁻¹ NGF antibodies (●) and GCM plus 500 ng ml⁻¹ NGF antibodies (▲). Results are expressed as percentages of surviving neurones $\pm$ s.e.m. ($n = 3$).

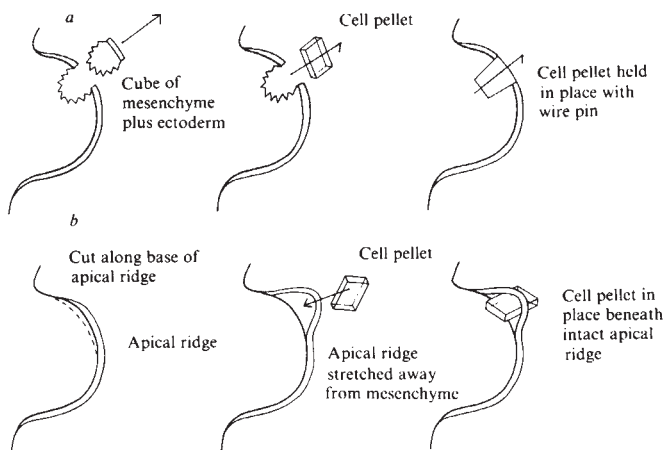


Fig. 1 Diagram to show how grafts of pieces of cell pellet were made to host wing buds (stage 20–21). The grafts were cut from a centrifuged pellet of cells which had been incubated for 1 h at 37 °C. The grafts were ~150–200 µm per side and contained about 6,000 cells (estimated from serial sections: average for 4 pieces = 5,979, s.d. 464). The cell suspensions were prepared from polarizing regions and equivalent-sized pieces of anterior mesenchyme tissue dissected from the wing buds of chick embryos (stage 22: Hamilton–Hamburger) following removal of the ectoderm with 2% trypsin (Difco). Various mixtures of polarizing region cells and inactive cells from anterior margins were made. For each suspension a total of 20 pieces of tissue was used. The standard grafting procedure, shown in *a*, involved removing a piece of mesenchyme plus overlying apical ridge from the anterior margin of the host limb bud opposite somite 16 or 17 and placing the piece of pellet in the hole. The graft was held in place by a pin of platinum wire. In the other grafting procedure, shown in *b*, a cut was made along the base of the apical ectodermal ridge over the anterior part of the host bud with a sharpened tungsten needle. The apical ectodermal ridge was raised up from the underlying mesenchyme and the piece of pellet was placed between. No mesenchyme tissue was removed. The apical ridge was stretched away from the mesenchyme and once the graft was in place it contracted back to hold the pellet in position.

Table 1 Effect of diluting polarizing cells with anterior margin cells on the digit formed next to the graft

% Of polarizing cells in pellet	No. of cases	Extra digit adjacent to graft				
		4	3	2	Knob	None
<i>a</i> , Standard grafting procedure shown in Fig. 1 <i>a</i>						
100	5	3	2	0	0	0
100 (50/50 wing/leg)	9	4	2	3	0	0
Total	14	7	4	3	0	0
55 (quail)	4	0	4	0	0	0
50	11	0	4	5	0	2
50 (quail)	6	0	1	3	2	0
Total	21	0	9	8	2	2
33	1	0	1	0	0	0
25	13	0	1	7	2	3
20	4	0	0	2	0	2
10	6	0	1	4	0	1
5	7	0	0	2	2	3
0	9	0	0	0	0	9
<i>b</i> , Grafts made to a slit beneath an intact apical ridge as shown in Fig. 1 <i>b</i>						
100	11	11	0	0	0	0
50	7	2	4	1	0	0
20	3	0	3	0	0	0
9-10	13	0	7	6	0	0
0	4	0	0	0	0	4

In the first set of experiments, cells from the polarizing region were mixed with inactive cells from the region along the anterior margin of the wing bud. By progressively diluting disassociated polarizing region cells with these inactive cells and grafting the mixture after re-aggregation to an anterior position in another bud, a clear relationship between the number of polarizing region cells and the digit formed next to the graft can be shown.

The results of grafts carried out by the standard procedure (Fig. 1a), but with varying proportions of re-aggregated polarizing region cells and inactive anterior cells, are listed in Table 1a. As the proportion of polarizing region cells in the grafts decreased, the digit formed next to the graft changed progressively: first a digit 3 (Fig. 2c) instead of a digit 4 (Fig. 2a) was produced, then a digit 2 (Fig. 2d) instead of a digit 3, and finally no additional digits at all (Fig. 2b) were specified. A

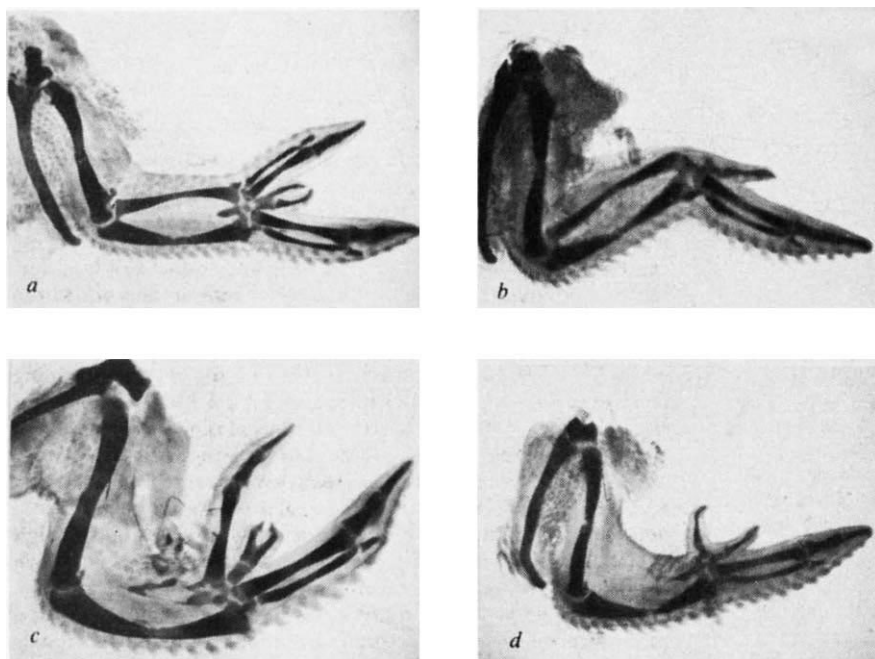


Fig. 2 Dorsal views of whole mounts of wings obtained following grafts of re-aggregated cells made as shown in Fig. 1a. *a*, 100% polarizing region cells. Pattern of digits is 4 3 2 2 3 4. *b*, 100% anterior margin cells. Pattern of digits is 2 3 4. *c*, 50% polarizing region cells and 50% anterior margin cells. Pattern of digits is 3 2 2 3 4. *d*, 5% polarizing region cells and 95% anterior margin cells. Pattern of digits is 2 2 3 4.

Table 2 Comparison of independent estimates of the number of polarizing region cells, placed under an intact apical ridge, which are required to specify each additional wing digit

	Digit 4	Digit 3	Digit 2
<i>a</i>	100(1)	79(10)	35(12)
<i>b</i>	164	64-32	32 or less

a, Mean numbers of polarizing cells on pieces of plastic film grafted under the apical ridge with the cells facing the ridge; *b*, number of polarizing cells on a face of a piece of pellet of re-aggregated polarizing and anterior mesenchyme cells grafted under an intact apical ridge. In *b*, data were calculated from the average number of cells on a face of a pellet and % polarizing cells in mixed pellet required to specify each digit. Values in parentheses indicate number of cases.

similar effect has also been obtained when polarizing region cells are diluted with BHK cells (unpublished observations). Even when only 5% of the cells were from the polarizing region, an additional digit 2 was obtained in one-third of the cases (Fig. 2*d*). Because the same order of loss of duplicate digits is found when the polarizing region is treated with increasing doses of γ -ray radiation⁴, it seems likely that the effect of the irradiation is to diminish the number of viable cells in the graft.

To investigate whether close contact with the apical ridge leads to more efficient signalling, as suggested by the recent work of Saunders⁵, another series of grafts, in which the polarizing region cells were progressively diluted with anterior mesenchyme cells, was carried out (Table 1b). In this series, however,

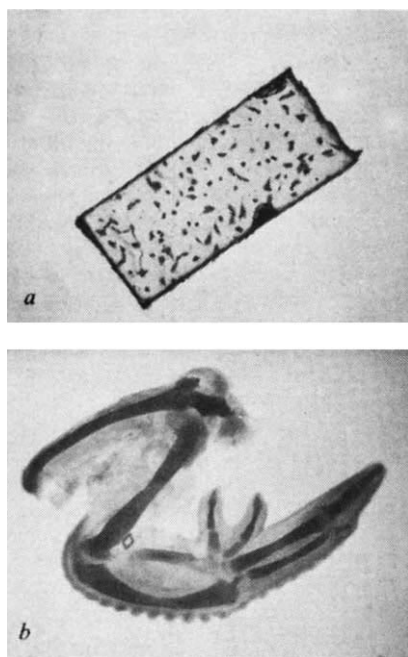


Fig. 3 *a*, Piece of plastic film (Melinex, ICI) after 3 h incubation on Parafilm (American Can Company) with a drop of suspension of polarizing cells prepared from at least eight polarizing regions. This piece of plastic (scale bar is 100 μm) has been fixed and stained with Leishman's to show attached cells, some of which have spread. To count the numbers of cells on such pieces of plastic (100–250 μm on a side) before grafting, the pieces were transferred with a spatula to a dish and viewed in an inverted phase-contrast microscope. The piece of plastic with a known number of polarizing cells was then carried into position over the host wing bud on a spatula and manoeuvred into the grafting site. *b*, Wing which developed after grafting of a piece of plastic film carrying 28 polarizing cells. The graft was made to a slit beneath the apical ridge and the film was oriented so that the cells faced the ridge. Digit pattern is 2 2 3 4. Note that the piece of plastic has ended up at the elbow of the developed wing.

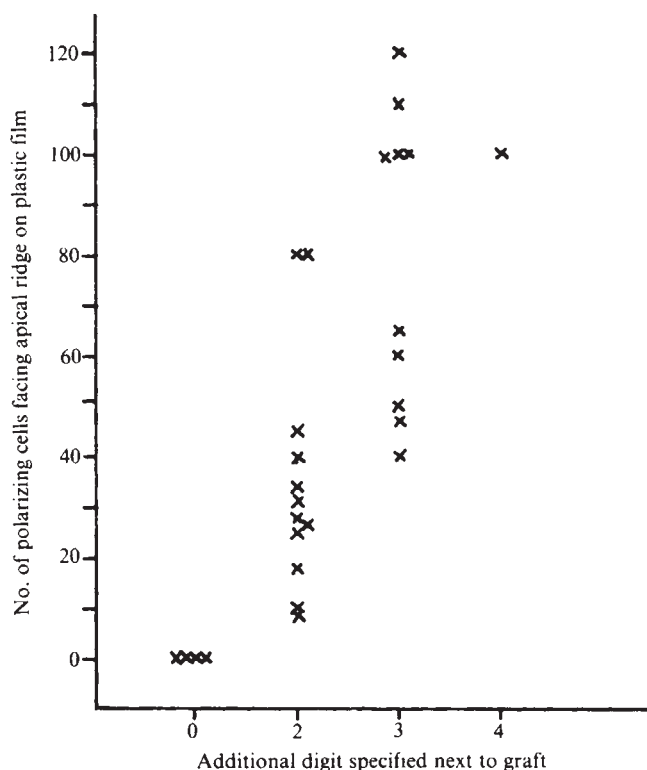


Fig. 4 Data on the digit which formed next to the graft after implantation of pieces of plastic film carrying varying numbers of polarizing cells. The plastic film was grafted to a slit beneath the apical ridge with the cells facing the ridge.

the apical ridge was left intact (Fig. 1*b*). It is clear that these grafts placed under an intact apical ridge are much more effective. For example, if the effects of grafts containing 50% polarizing region cells are compared, digit 4 was still specified in some cases when grafts were placed under the ridge, whereas an extra digit 4 has never been obtained with a standard graft.

From the total numbers of cells in pieces of pellet the following estimates can be made: with the standard grafting procedure, at least 6,000 polarizing region cells (100% polarizing region cells in the graft) are required to obtain digit 4 next to the graft; to obtain digit 3, 3,000–6,000 cells are required, and for digit 2 between 3,000 and as few as 300 are needed. For grafts made under an intact apical ridge, a digit 4 requires about 3,000 polarizing region cells, but only about 600 cells are required for a digit 3 and even fewer for a digit 2.

In the second set of experiments, small numbers of polarizing cells stuck to pieces of plastic film (Fig. 3a) were implanted just beneath the apical ridge. Figure 4 shows that remarkably small numbers of polarizing cells can now specify additional digits in the chick wing (Fig. 3b). Grafts of pieces of plastic with small numbers of anterior mesenchyme cells (five cases) always resulted in normal wings. Figure 4 also shows a clear relationship between the number of polarizing cells grafted on the piece of film and the digit which developed next to the graft. The estimate of the mean number of cells required to specify each wing digit is shown in Table 2. Thus, to specify digit 4 at least 100 polarizing cells must be grafted beneath the apical ridge, whereas specification of digit 3 requires 79 cells and digit 2, 35 cells. In one case, the transfer of only nine polarizing cells led to a wing with an additional digit 2.

The two independent estimates of the number of polarizing cells in contact with the ridge that are required to specify each additional wing digit are apparently very different. However, if the number of polarizing cells on only the face of each mixed pellet, used for grafts under the apical ridge, are considered, the number of cells required for signalling agrees well with the

estimates obtained from implanting known numbers of cells on pieces of plastic film (Table 2). Thus, it seems likely that the signalling ability of the polarizing region involves only a monolayer of cells, the rest of the cells in a grafted pellet being superfluous or inactive. Both this finding and the observation that the mixed aggregates signal best when grafted under an intact apical ridge suggest that cooperation between the monolayer of polarizing cells and the apical ectodermal ridge is essential for positional signalling.

This work was supported by the MRC. I thank Professors L. Wolpert and B. Alberts for their enthusiastic encouragement. I also thank Professor Alberts for two visits to his laboratory supported by NIH grant GM23928, and M. Goodman and A. Crawley for help with photography and histology.

Received 14 August; accepted 21 November 1980.

1. Saunders, J. W. & Gasseling, M. T. in *Epithelial-Mesenchymal Interactions* (eds Fleishmajer, R. & Billingham, R. E.) 78-97 (Williams & Wilkins, Baltimore, 1968).
2. Tickle, C., Summerbell, D. & Wolpert, L. *Nature* **254**, 199-202 (1975).
3. Tickle, C. in *Development in Mammals* Vol. 4 (ed. Johnson, M. H.) 101-137 (North-Holland, Amsterdam, 1980).
4. Smith, J. C., Tickle, C. & Wolpert, L. *Nature* **272**, 612-613 (1978).
5. Saunders, J. W. in *Vertebrate Limb and Somite Morphogenesis* (eds Ede, D. A., Hinchliffe, J. R. & Balls, M.) 1-24 (Cambridge University Press, 1977).

Flow cytometry analysis of T cells and continuous T-cell lines from autoimmune MRL/l mice

D. E. Lewis, J. V. Giorgi & Noel L. Warner

Immunobiology Laboratories, University of New Mexico School of Medicine, Albuquerque, New Mexico 87131

The study of spontaneous autoimmunity in mouse models affords an opportunity to determine the cellular basis of the immune dysregulation observed in this disease. Recently, a new mouse strain, MRL/Mp-*lpr/lpr* (MRL/l) has been developed¹ which carries an autosomal recessive gene (*lpr*) that results in massive lymph node enlargement concomitant with the development of several autoantibodies¹⁻⁴. The interest in this strain lies in the possibility that the defect in T-cell regulation of the immune response is manifested at a different level from that in the NZB mouse³. It has been reported that the proliferating population of lymphoid cells in the nodes of these mice are T cells, but that many of them are devoid of Lyt surface antigens⁴. We have accordingly initiated several lines of research with these mice, including quantitative flow cytometry characterization of Lyt antigen expression of cells in the lymph nodes of the mice. In an approach to isolate and study the properties of these cells, we have also established continuous cell lines from the lymph node cells of MRL/l mice, using techniques similar to those used to establish continuous lines of antigen-activated cytotoxic T cells^{5,6} and helper T-cell populations⁷.

Lymph node cells from MRL/l and MRL/n mice at various ages, and T-cell lines which were established from MRL/l lymph node cells were examined for Lyt-1, Lyt-2 and Thy-1.2 cell surface density using indirect immunofluorescence. Xenogeneic (rat) hybridoma-derived monoclonal antibodies which have specificities for Lyt-1, Lyt-2 (both framework determinants) and Thy-1.2, were provided by Drs Ledbetter and Herzenberg of Stanford University⁸. The lymph node cells were stained for 20 min on ice, washed three times in Eisen's balanced salt solution (BSS) and then fluorescein-conjugated (Fl) goat anti-rat immunoglobulin was added as a second step reagent. The cells were incubated for an additional 20 min, washed three times in BSS and examined using the flow cytometry systems at Los Alamos Scientific Laboratories^{9,10} or a Becton Dickinson FACS-III cell sorter.

Table 1 Comparison of cell-surface markers on *in vivo* MRL/lpr lymph node cells and *in vitro* MRL/lpr cell lines

Surface marker	MRL/lpr MLN cells	MRL/lpr T-cell line
Thy-1.2	Moderate density (lower than MRL/n)	Moderate density
Lyt-1	Increased % positive Lower density	Most positive Low density
Lyt-2	Marked reduction in % positive	Most lines variable
Lyt-3	As for Lyt-2	As for Lyt-2
T-30	Lower density	Most lines positive Low density
Th-B	Negative	Negative
FcR	Negative	Negative

Surface marker was detected by flow cytometry with monoclonal antibodies. Results for MRL/lpr are intensity and per cent cells relative to age matched control MRL/n mice. The MRL/lpr T-cell line is representative of most MRL/lpr cell lines. MLN, mesenteric lymph node.

Attempts were made to establish continuous cell lines from MRL/l, MRL/n and C57BL/6 mesenteric lymph node cells by plating 5×10^5 to 1×10^6 cells ml^{-1} in 5-ml volumes of Dulbecco's modified Eagle's medium (DMEM) supplemented with non-essential amino acids (1 ml 100 ml^{-1} , 100x, Gibco), 10^{-4} M 2-mercaptoethanol, 10% fetal calf serum and 25% T-cell growth factor (TCGF) (v/v), which was prepared from rat spleen cells stimulated with $5 \mu\text{g ml}^{-1}$ concanavalin A (Con A) for 46 h (ref. 5).

The results of the analysis of Lyt phenotypes of lymph node cells from MRL/l and MRL/n mice are shown in Fig. 1. The

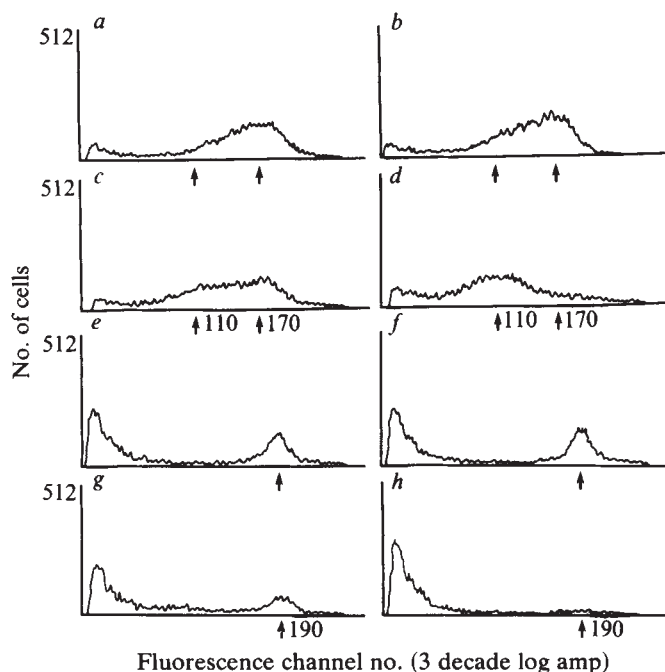


Fig. 1 Flow cytometry analysis of Lyt-1 and Lyt-2 expression on mesenteric lymph node cells of MRL/lpr and MRL/n mice. In each analysis cell suspensions were stained with the hybridoma-derived supernatants followed by fluorescein-conjugated SJL anti-rat immunoglobulin. The left column of panels (a, c, e, g) shows cell suspensions from 2-month old mice, and the right-hand column of panels (b, d, f, h) those from 5-month old mice. a, b, MRL/n cells stained with anti-Lyt-1; c, d, MRL/lpr with anti-Lyt-1; e, f, MRL/n with anti-Lyt-2; g, h, MRL/lpr with anti-Lyt-2. The key distinction between MRL/lpr and MRL/n mice is the presence of a low density Lyt-1 peak around channel 110, and a selective reduction in Lyt-2 cells in older MRL/lpr mice.

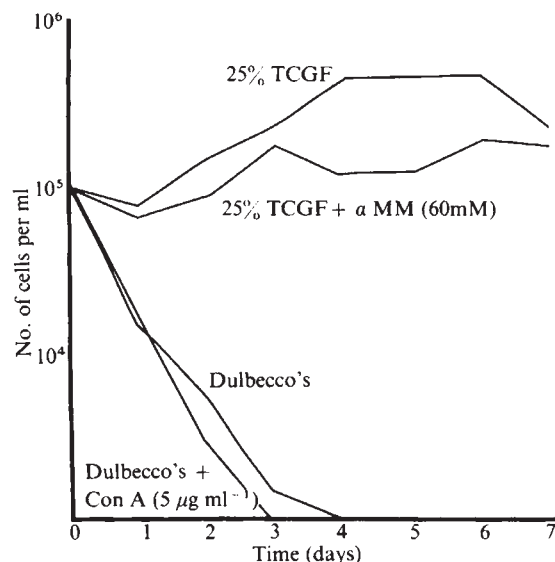


Fig. 2 Dependence on T-cell growth factor (TCGF) of MRL-III continuous T-cell line. Four aliquots of MRL-III cells were seeded at 10^5 ml^{-1} in the presence of Dulbecco's medium with the indicated additive. Growth occurs in the presence of 25% TCGF either with or without α -methylmannoside. Growth of the line is not supported by the addition of Con A alone.

MRL/l lymph node population at 8 weeks of age is characterized by a shift in the surface density of Lyt-1, in that, compared with MRL/n lymph node cells, a population of cells with lower Lyt-1 expression is evident. By 5 months of age this low surface density population of Lyt-1 cells has become the predominant population and by 5 months of age there are very few Lyt-2-positive lymph node cells (that is, either Lyt-1⁺2⁺ or Lyt-1⁺2⁻). The Thy-1.2 staining characteristics indicate that the MRL/l mouse lymph node cells, which are all Thy-1.2 positive, have lower amounts of Thy-1.2 antigen than do MRL/n lymph node cells. As the cell population is also somewhat larger, the actual cell-surface density is considerably lower than in the MRL/n mice.

Seven MRL/l cell lines have now been established and several of the lines have been in culture for up to 8 months. The lines were all established from mesenteric lymph node cells from 3–5-month old MRL/l mice. The cells will not grow in DMEM without TCGF, nor will they grow in DMEM supplemented with $5 \mu\text{g ml}^{-1}$ Con A (Fig. 2). Furthermore, addition of 60 mM α -methylmannoside to the growth medium does not inhibit the effect of the TCGF. These data indicate that the growth of the MRL/l cell lines is dependent on a growth factor in the TCGF medium which is not Con A itself.

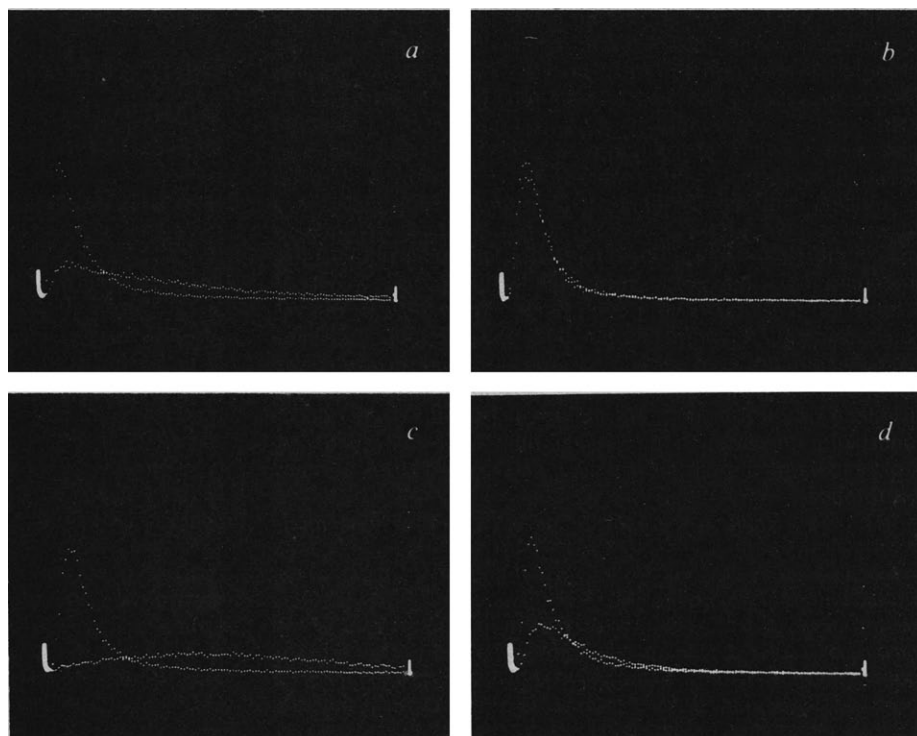
Attempts made to establish continuous cell lines from MRL/n and C57BL/6 lymph node cells were unsuccessful. The cells multiplied much more slowly than the cells from MRL/l mice, and never reached high cell densities *in vitro*. These lymph node cells were occasionally maintained in culture for periods of up to 2 months.

Because it is important to know whether these *in vitro* continuous T-cell lines are representative of the abnormal proliferating cells *in vivo*, we have compared the *in vivo* and *in vitro* cell lines using a range of surface markers. After several weeks in culture, the *in vitro* lines show a complete absence of B cells, and all cells are Thy-1.2 positive. The Lyt phenotype of the cells has fluctuated, however, and as shown in Fig. 3, includes both Lyt-1⁺2⁻ and Lyt-1⁺2⁺ lines. The predominant phenotype of most of the lines has been Lyt-1⁺2⁻, with fluctuations primarily among cells that are Lyt-2⁺. Some lines thus contain many Thy-1⁺ Lyt-null cells. These may, however, have lower undetectable amounts of Lyt-1.

Using a broader range of cell-surface markers, we show in Table 1 that the *in vitro* lines, as studied here, are generally similar to the predominant population of cells *in vivo*.

Our results indicate that the massive lymphoproliferation observed in MRL/l mice is associated with a decline in the proportion of Lyt-2-positive T cells. This population includes both Lyt-123- and Lyt-23-positive cells. The majority of the cells present in these large nodes are Lyt-1 positive, but have a distinctly lower density of Lyt-1 on their surfaces than normal cells. These results are in contrast to those of Theofilopoulos *et al.*⁴, who reported that about 50% of the cells in the MRL/l lymph node are Lyt-null T cells, although they did suggest that the 'Lyt-null cells' may not be completely devoid of Lyt antigens. With flow cytometry we find only approximately 10%

Fig. 3 Flow cytometry analysis of Lyt-1 and Lyt-2 expression on two MRL continuous T-cell lines. Each panel shows the fluorescence profile (FACS-III histogram) of the cell suspension stained with either medium or hybridoma supernatant, followed by fluorescein-conjugated goat anti-rat immunoglobulin. Panels *a* and *b* show cell line MRL-VI stained, respectively, with anti-Lyt-1 and anti-Lyt-2, and panels *c* and *d* cell line MRL-VII stained, respectively, with anti-Lyt-1 and anti-Lyt-2. In each panel the number of cells is on the ordinate and the relative fluorescent intensity on the abscissa.



of the T cells in the MRL/l lymph node to be of Lyt-null type (but Thy-1.2 positive). Thus, this quantitative analysis clearly indicates that the proliferating T-cell population in the MRL/l lymph node may be a relatively homogeneous T-cell subset. Further analysis of these cells with other differentiation antigens is proceeding.

The present report also extends the findings of other investigators which indicate that continuously proliferating lines of T cells can be readily established in long-term culture as long as T-cell growth factors are present. Our results indicate that it is possible to establish cells from MRL/l mice (but not cells from normal mice) in continuous culture without deliberate exogenous antigenic stimulation. Thus, it might be proposed that antigenic stimulation for these lymph node cells from MRL/l mice is from an autoantigenic source, probably derived *in vivo*

before culture, as the lines do require continual TCGF for maintenance.

It is interesting that the cell lines generated from the lymph nodes of MRL/l mice are variable in their Lyt expression, although all are T cells. Cloning and cell sorting experiments are in progress to resolve this problem and to determine whether this variability truly represents instability in Lyt expression in a continuously proliferating clonal line. The fact that cell lines can be established at all in the absence of antigenic stimulation may allow the development of pure subpopulations of specific Lyt antigen-bearing cells for further dissection of the functional role of Lyt subsets in the aberrant immune response observed in these autoimmune mice.

This work was supported by NIH research grants AM19841 and fellowships AI05779 and CA05921. We thank Mrs Jerri Davis for maintenance of the MRL mouse colonies.

Received 7 May; accepted 24 October 1980.

1. Murphy, E. D. & Roths, J. B. *Fedn Proc.* **36**, 1246 (1977).
2. Murphy, E. D. & Roths, J. B. in *Genetic Control of Autoimmunity* (eds Rose, N. R., Bigazzi, P. E. & Warner, N. L.) 207-221 (Elsevier, New York, 1978).
3. Gershon, R. K., Horowitz, M., Kemp, J. D., Murphy, D. B. & Murphy, E. D. in *Genetic Control of Autoimmunity* (eds Rose, N. R., Bigazzi, P. E. & Warner, N. L.) 223-227 (Elsevier, New York, 1978).
4. Theofilopoulos, A. N., Eisenberg, R. A., Bourdon, M., Crowell, J. S. Jr & Dixon, F. J. *J. exp. Med.* **149**, 516-534 (1979).
5. Gillis, S. & Smith, K. A. *Nature* **268**, 154-156 (1977).
6. Nabholz, M., Engers, H. D., Callovo, D. & North, M. *Curr. Topics Microbiol. Immun.* **81**, 176 (1978).
7. Watson, J. A., Aarden, L. A. & Lefkowitz, I. J. *Immun.* **122**, 209 (1979).
8. Dennert, G. *Nature* **277**, 476-477 (1979).
9. Shreier, M. H. & Tees, R. *Int. Archs Allergy appl. Immun.* **61**, 227-237 (1980).
10. Ledbetter, J. A. & Herzenberg, L. A. *Immun. Rev.* **47**, 63-90 (1979).
11. Steinkamp, J. A. *et al. Devs scient. Instrum.* **44**, 1301-1310 (1973).
12. Warner, N. L., Daley, M. J., Richey, J. & Spellman, C. *Immun. Rev.* **48**, 197-243 (1979).

Cyclosporin A promotes spontaneous outgrowth *in vitro* of Epstein-Barr virus-induced B-cell lines

A. Graham Bird^{*‡}, Sandra M. McLachlan[†] & Sven Britton^{*}

^{*} Department of Immunobiology, Wallenberg Laboratory, Karolinska Institute, S-104 05 Stockholm, Sweden and Department of Infectious Diseases, Danderyd Hospital, S-182 03 Danderyd, Sweden

[†] Department of Medicine, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne NE1 7RU, UK

Cyclosporin A (CSA) is a fungal metabolite which exerts profound effects on the immune system and has potential as a selective immuno-suppressive agent¹. Clinical trials with human renal allograft recipients have confirmed this potential but there have been disturbing reports of lymphoma in a significant number of patients^{2,3}. Despite extensive animal studies, the specificity of this drug for human lymphocyte subpopulations is largely unknown⁴. We demonstrate here that *in vitro* CSA has no apparent effect on Epstein-Barr virus (EBV)-induced B-lymphocyte activation, but totally inhibits the T-cell dependent pokeweed mitogen (PWM) B-cell response. In addition, CSA markedly facilitates the outgrowth of B-lymphoblastoid cell lines from both EBV-infected and non-infected lymphocytes of EBV immune donors cultured *in vitro*. These results indicate that CSA can interfere with the lymphocytes normally responsible for maintaining the life-long carrier state initiated by primary infection with EBV, allowing outgrowth of the persistently infected cells circulating in the peripheral blood.

We have investigated the effects of CSA on human polyclonal B-cell activation *in vitro*. Figure 1 demonstrates the differential effects of CSA on activation induced by two mechanistically different activators, EBV and PWM. The results clearly show that CSA completely inhibits PWM-induced B-cell activation which is T-cell and monocyte dependent^{5,6} whereas the polyclonal activation produced by the T- and monocyte-independent EBV⁶ is actually enhanced early in the culture period at 6 days. CSA did not influence cell viability in the PWM cultures but markedly reduced morphological blast transformation.

We next studied the effects of CSA on the generation of lymphoblastoid cell lines from individuals of known EBV immune status whose lymphocytes were either infected *in vitro* with EBV or uninfected. Table 1 summarizes the results obtained from a number of individuals. In cultures of lymphocytes not treated with CSA, lymphoblastoid cell lines were only obtained from cultures to which EBV had been added. In agreement with the results of others^{7,8}, cell lines were easily obtained from lymphocytes from a seronegative donor infected

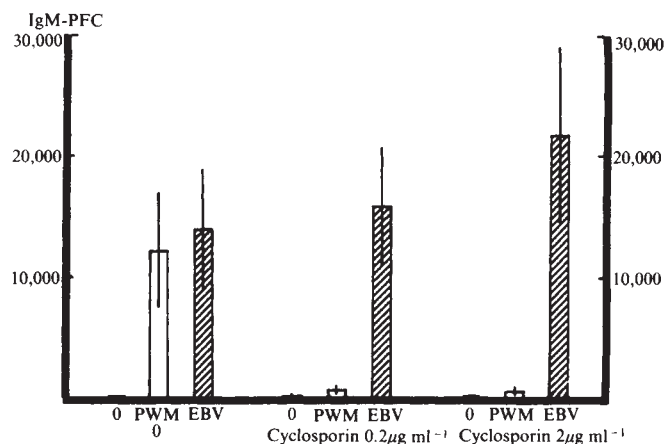


Fig. 1 The effects of cyclosporin A on the polyclonal activation of human blood lymphocytes by EBV and PWM. Lymphocytes from seven adult volunteers of known EBV immune status were cultured at a final density of 10^6 ml^{-1} in RPMI 1640 medium supplemented with 10% human AB serum (anti-VCA positive) and antibiotics. Cultures were incubated for 6 days in 50-ml plastic flasks (Falcon 3013) in the presence of 5% CO_2 . EBV infection was achieved by 1 h pre-incubation at 37°C of a suspended pellet of 5×10^6 lymphocytes with 1 ml of B95-8 cell line supernatant. PWM was added to appropriate cultures at a final concentration of $20 \mu\text{g ml}^{-1}$. Cyclosporin A (Sandoz) was initially dissolved in a small volume of 95% ethanol and then diluted in Earle's balanced salt solution to give a concentration of $100 \mu\text{g ml}^{-1}$. The drug was added to cultures to give a final concentration of 2.0 or $0.2 \mu\text{g ml}^{-1}$. Cultures were assayed for the presence of antibody-secreting cells by use of the protein A plaque assay which has been described elsewhere⁶. Results are expressed as IgM plaque-forming cells (PFC) per 10^6 viable cells at the end of culture. A similar relation was found for IgG PFC (data not shown).

[‡] To whom correspondence should be addressed at the Department of Immunology, The Medical School, Vincent Drive, Birmingham B15 2TJ, UK.

Table 1 Effect of cyclosporin A on the *in vitro* outgrowth of lymphoblastoid cell lines from individuals of known EBV-immune status

Donor	Serological status (anti-VCA)	CSA addition (2 µg mg ⁻¹)	EBV infection <i>in vitro</i>	Cell lines established
1-6	+	+	+	+
		—	+	—
		+	—	+
7-8	+	—	—	—
		+	+	+
		—	+	—
9	+	+	—	—
		—	+	+
		+	—	+
10	—	—	—	—
		+	+	+
		—	+	—

Immune status of the donors was examined by determination of the antibody titre to EB-viral capsid antigen (VCA). Cultures were established as in Fig. 1 and were regularly examined for cell line formation. This was determined visually by the appearance of progressively growing foci of transformed cells⁷. The scores above are from 4–12 weeks of culture. In many cases the presence of lines was confirmed by transferring the cells into secondary cultures. Cell lines arising in non-EBV-infected lymphocytes were examined for the presence of EBNA by the method of Reedman and Klein⁹.

with EBV, but, despite initial transformation in all seropositive donor cells, regression of cell lines occurred in eight out of nine donor cultures. Addition of CSA allowed cell lines to be established by 4 weeks with ease and without regression from all seropositive donors so far tested. More significantly, B-cell lines have also been obtained from cultured lymphocytes from six out of nine seropositive donors exposed to CSA but not infected with EBV. These cell lines contained the EB-determined nuclear antigen (EBNA), a characteristic of EBV transformation⁹ and they were of similar morphology to those derived from cells exposed to EBV (K. Nilson, personal communication).

These results clearly demonstrate that CSA exerts profoundly selective effects on human lymphocyte subpopulations *in vitro*. The enhancement of the polyclonal immunoglobulin response induced by EBV and abrogation of that by PWM strongly suggest that at least the fraction of B cells activatable by EBV is refractory to the action of CSA. We interpret the abolition of PWM responsiveness to mean that the site of action of CSA is the T lymphocyte or, less likely, the monocyte—both essential for a PWM-induced polyclonal immunoglobulin response.

Furthermore, CSA seems to promote the early and complete outgrowth of EBV B-lymphoblastoid cell lines from immune donors *in vitro*. It has been shown that at a cell density of 10⁶ ml⁻¹, lymphoblastoid cell line generation from EBV-infected lymphocytes from immune donors is inhibited by the *in vitro* appearance of immune T lymphocytes⁷. It seems that CSA specifically deletes or functionally inactivates these T cells, allowing the rapid appearance of EBV-transformed cell lines from all immune donors tested. In six out of nine seropositive individuals, EBV-bearing cell lines have been directly established from the blood merely by the addition of CSA alone. It is known that EBV-infected B cells persist in the lymphoid system of immune donors and can grow out into lines in appropriate conditions if T lymphocytes are removed⁷. CSA seems to facilitate this outgrowth, probably by its specific action on the T lymphocyte.

The experimental conditions we have used are certainly unfavourable for *de novo* infection of B cells by *in vitro* released infectious particles¹⁰, because cells were grown in human AB

anti-VCA positive serum. In the presence of specific antibody EBV cannot activate B lymphocytes¹¹. Although we cannot exclude transfer of virus between cells in intimate contact we suggest that the lymphoblastoid cells growing out from CSA-treated blood of EBV immune donors really represent the progeny of the latently infected cell.

There have been recent disturbing reports of lymphomas presenting in renal allograft recipients treated with CSA and there is a general tendency for anti-EBV titres to rise in patients on treatment with CSA. In one reported case lymphoma followed asymptomatic infectious mononucleosis¹² and in another EBNA was present in the lymphomatous B cells³. On the basis of these *in vitro* experiments we suggest that in the presence of CSA, T-lymphocyte surveillance of EBV-infected B cells is no longer effective. The result is a polyclonal proliferation of EBV-transformed B cells which *in vitro* from B-lymphoblastoid lines but *in vivo* manifest as the polyclonal immunoblastic proliferation characteristic of immunosuppressed patients¹³.

We thank Kicki Hild for technical assistance, Kenneth Nilson for characterizing the lines and G. Klein for supplies of B95-8 supernatant. This work was supported by the Swedish MRC and in part by a grant from the North of England Cancer Research Campaign.

Received 2 September; accepted 7 November 1980.

- Borel, J. F., Feurer, C., Mognée, C. & Stähelin, H. *Immunology* **32**, 1017–1025 (1977).
- Calne, R. Y. *et al. Lancet* **ii**, 1033–1036 (1979).
- Crawford, D. H. *et al. Lancet* **i**, 1355–1356 (1980).
- Leoni, P., Garcia, R. C. & Allison, A. C. *J. clin. Lab. Immun.* **1**, 67–72 (1978).
- Janossy, G., Gomez de la Concha, E., Waxdal, M. J. & Platts-Mills, T. *Clin. exp. Immun.* **26**, 108–117 (1976).
- Bird, A. G. & Britton, S. *Scand. J. Immun.* **9**, 507–510 (1979).
- Moss, D. J., Rickinson, A. B. & Pope, J. H. *Int. J. Cancer*, **22**, 622–668 (1978).
- Thorley-Lawson, D. A., Chess, L. & Strominger, J. L. *J. exp. Med.* **146**, 495–598 (1977).
- Reedman, B. M. & Klein, G. *Int. J. Cancer* **11**, 499–520 (1973).
- Rickinson, A. B., Finerty, S. & Epstein, M. A. *Int. J. Cancer* **19**, 775–782 (1977).
- Bird, A. G. & Britton, S. *Immun. Rev.* **45**, 541–567 (1979).
- Nagington, J. & Gray, J. *Lancet* **i**, 536–537 (1980).
- Purtillo, D. T. *Lancet* **i**, 300–303 (1980).

Inhibition of *P. falciparum* growth in human erythrocytes by monoclonal antibodies

L. H. Perrin, E. Ramirez, P. H. Lambert & P. A. Miescher

WHO Research and Training Centre, Department of Medicine, Hôpital Cantonal, 1211 Geneva 4, Switzerland

Malaria is increasing in incidence and prevalence in most tropical areas and is a major problem for both individuals and communities¹. Current malaria research is aimed at developing vaccines^{2–4} and, for this, it may be useful to define *Plasmodium* antigen(s) related to the development of a protective immune response in the host. Monoclonal antibodies have recently been shown to interfere with rodent malaria infection (*Plasmodium berghei*) at the sporozoite⁵ or merozoite⁶ stage. We have now raised monoclonal antibodies against single antigenic determinant(s) of *Plasmodium falciparum* and report that some of them inhibit the growth of erythrocytic forms of *P. falciparum* *in vitro*.

Red blood cells (RBCs) parasitized with *P. falciparum* were obtained from a European patient on his return from Senegal and were adapted and maintained in *in vitro* culture according to

Table 1 Inhibition of *P. falciparum* growth by monoclonal antibodies

Culture medium + 10% ascitic fluid from various hybridomas	Ig class	% Parasitaemia		% Growth inhibition
		Total	Intact parasites	
Hb28 cl1	IgG3	0.2	0	100
Hb26 cl3	IgG2a	1.5	0.6	96
Hb31 cl2	IgG3	1.2	0.5	95
Hb12 cl4	IgG1	9.6	9.4	19
Hb9 cl1	IgM	9.7	9.5	18
Medium + 5% NMS		9.8	9.6	17
Medium + 10% NaCl		11.8	11.4	0
P3-X63-Ag8 (Fo)		9.6	9.4	19

The inhibition of *P. falciparum* growth *in vitro* by various monoclonal antibodies was analysed by a modification of the technique described by Wilson and Phillips¹⁴. Synchronized cultures of ring forms of *P. falciparum* were diluted with uninfected human RBCs to provide a final suspension of 1.5% RBCs and 0.5% parasitaemia. Aliquots (150 µl) of this suspension were placed in 96 flat-bottomed wells (3040 Falcon plates). Each microculture was done in triplicate. The medium used for the culture (RPMI 1640 with 10% normal human serum)⁷ was supplemented with either 10% ascitic fluid produced by various hybridomas or as control with 5% serum of normal BALB/c mice (NMS), 10% isotonic NaCl, or ascitic fluid induced by myeloma cells used for fusion. The culture medium was removed from the settled cells each day and replaced with fresh medium. The plates were incubated at 37 °C in a candle jar⁹. Parasitaemia was determined on Giemsa-stained thin blood smears after 80 h of incubation. In these conditions, more than two cycles of replication took place in the wells containing the medium +10% NaCl. At the end of the experiment, some of the parasites were pyknotic, especially those in cultures supplemented with Hb28 cl1, Hb26 cl3 and Hb31 cl2. Therefore, the percentage inhibition was determined over the number of intact parasites. The results reported here represent three hybridomas with inhibitory activity and two out of a group of eight hybridomas without any significant inhibitory activity (10–19%).

the method of Trager and Jensen⁷. These cultures were not synchronized and contained all the various developmental erythrocytic stages (rings, trophozoites, schizonts, merozoites). Synchronization was induced by two treatments with 5% mannitol at an interval of 34 h (ref. 8); this treatment lysed the mature forms (trophozoites and schizonts), leaving only the ring forms. Approximately 30 h after treatment with mannitol, merozoites were released from schizonts and non-infected RBCs were invaded.

In the first series of experiments, 8-week-old female BALB/c mice were immunized intraperitoneally (i.p.) three times at 2-week intervals with 100 µg of merozoite and schizont antigen in Freund's incomplete adjuvant. Four days before the fusion experiment a booster of 5 µg of the antigenic preparation was administered intravenously (i.v.). Merozoites and schizonts were purified from a synchronized culture of *P. falciparum* by Plasmagel sedimentation⁹ and contained <10% normal RBCs. In a second series of experiments, BALB/c mice were infected i.p. with 1×10^5 erythrocytic forms of *P. berghei*, treated with chloroquine¹⁰ and 3 weeks later injected i.v. with 5×10^5 erythrocytic forms of *P. berghei* 4 days before the fusion procedure. For each experiment, 2×10^7 immune spleen cells in suspension were fused by polyethylene glycol with 1×10^7 non-secreting mouse myeloma cells derived from P3-X63-Ag8 (clone FO given by Dr FASEKAS, Basel Institute of Immunology). Conditions for fusion were essentially as described by Galfre *et al.*¹¹. Positive hybrids were clone purified twice on mouse macrophage monolayers by limiting dilution and further grown in T75 Falcon flasks. 5×10^6 cells were injected i.p. into BALB/c mice which had been primed 1 week previously with 0.5 ml Pristan i.p.

An indirect immunofluorescence technique was used to assay hybrid supernatants. Acetone-fixed *P. falciparum*-parasitized human RBCs or *P. berghei*-parasitized mouse RBCs were used as antigenic preparation and rabbit fluoresceinated anti-mouse F(ab')₂ as the developing second antibody^{10,12}. Antibodies specific for the surface antigen or merozoites were similarly detected by reacting hybrid supernatants or ascitic fluid with a suspension of unfixed schizonts and merozoites ($\sim 10^5$ per 25 µl) purified by Plasmagel⁹.

Spleen cells from nine mice immunized with *P. falciparum* merozoites and schizonts and two mice immunized with *P. berghei* were fused in 11 experiments: 47 of 978 supernatants tested reacted with *P. falciparum*-parasitized RBCs; two of these supernatants obtained from mice immunized with *P. berghei* reacted with both *P. berghei* and *P. falciparum*. From each fusion experiment, hybrid cells from one or two wells (total 15) were selected, clone purified twice on macrophage monolayers and grown in large amounts. The cells were injected i.p. into mice, and the ascitic fluid was collected. When the 15 ascitic fluid samples were tested by indirect immunofluorescence against the different developmental stages of acetone-fixed parasites, 10 samples (including Hb28 cl1 and Hb31 cl2) reacted with trophozoites and schizonts only, two (including Hb26 cl3) reacted with schizonts only, and three reacted with all stages. The ascitic fluid samples were positive by indirect fluorescence at dilutions of 1/5,000–1/100,000.

The culture medium used for *P. falciparum* culture⁷ was supplemented with 10% of the various ascitic fluids and the mixtures were tested for their ability to inhibit the multiplication of erythrocytic forms of *P. falciparum*. An 80-h assay corresponding to more than two cycles of multiplication in normal culture conditions was used. Three out of 11 ascitic fluids tested had an inhibitory activity, two of them, Hb26 cl3 and Hb31 cl2, were produced with spleen cells from mice immunized against *P. falciparum* antigens, and the third one, Hb28 cl1, with spleen cells from mice infected with *P. berghei* (Table 1). Sera from

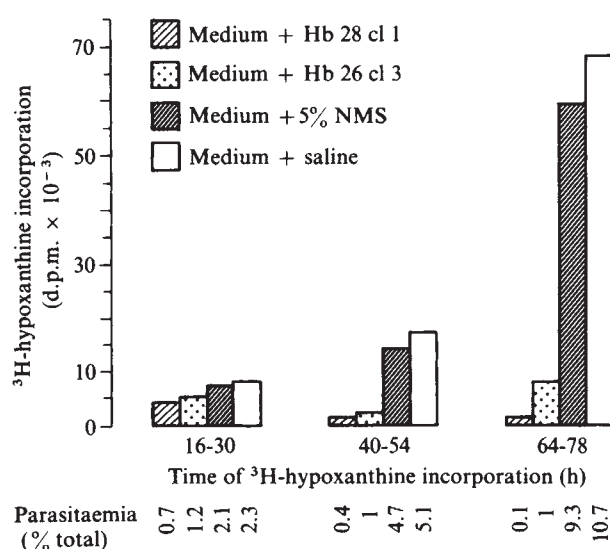


Fig. 1 Incorporation of ³H-hypoxanthine was determined in *P. falciparum* cultures supplemented with 10% ascitic fluid from Hb26 cl3 and Hb28 cl1 hybridomas, 5% BALB/c normal serum and 10% isotonic NaCl, three times during an interval of 14 h. The experimental design was identical to that described in Table 1 legend, except that 10 µl of ³H-hypoxanthine (1 µCi) were added to each culture at the beginning of the labelling period ($18.5\text{--}55.5 \times 10^{-3}$ Bq mmol⁻¹ specific activity, NEN). The percentage parasitaemia was determined at the end of each labelling experiment on Giemsa-stained thin blood smears.

Table 2 Specificity of the inhibition of *P. falciparum* growth

Culture medium supplemented with:	% Parasitaemia	% Growth inhibition
1. Hybridoma Hb26 cl3		
10% ascitic fluid	0.8	91
IgG-depleted 10% ascitic fluid	7.2	15
Purified IgG fraction (0.5 mg ml ⁻¹)	0.9	89
2. Mouse immunoglobulins		
IgG2a (LPC-1) (1 mg ml ⁻¹)	7.8	7
IgG2b (MOPC-195) (1 mg ml ⁻¹)	9.1	0
Normal mouse IgG (1 mg ml ⁻¹)	7.7	9
3. Medium + 10% NaCl 0.9%	8.4	0

The specificity of the inhibition of *P. falciparum* growth was analysed in experimental conditions identical to those described in Table 1 legend. The IgG fraction from Hb26 cl3 ascitic fluid was purified by 50% ammonium sulphate precipitation and DEAE cellulose chromatography and shown to be devoid of contaminants when analysed by immunoelectrophoresis. As a control, Hb26 cl3 ascitic fluid was depleted of IgG by passage on a Sepharose-protein A column (Pharmacia) and reconstituted to its original volume by negative pressure dialysis. Mouse immunoglobulin was purified from normal BALB/c sera and the IgG2a and IgG2b from the ascitic fluid of BALB/c mice bearing LPC-1 and MOPC-195 myeloma tumours. These immunoglobulins were prepared by precipitation in 50% ammonium sulphate followed by DEAE column chromatography. The concentration indicated in parentheses for IgG is the final concentration in the medium.

mice injected with hybrid cells of Hb26 cl3 and Hb28 cl1 were also tested and gave, respectively, 78% and 99% inhibition of *P. falciparum* growth when added at a final concentration of 5% to the culture medium.

Hb26 cl3, Hb28 cl1 and Hb31 cl2 monoclonal antibodies inhibited the development of schizonts and the invasion of normal RBCs by merozoites. Normal development of ring forms into trophozoites was observed when differential counts of the developmental stages were monitored on synchronized cultures supplemented with either normal medium or medium containing 10% ascitic fluid from each of the inhibitory monoclonal antibodies. Subsequent maturation of trophozoites into schizonts occurred; however, the schizonts frequently showed morphological signs of degeneration in cultures supplemented with any one of the three monoclonal antibodies. (At the end of the first cycle, these degenerated schizonts represented 15–35% of the expected number of schizonts.) In normal culture, 7.6 new ring forms were generated in the second cycle from each late trophozoite and schizont present in the previous cycle. However, in cultures supplemented with Hb26 cl3, Hb28 cl1 or Hb31 cl2, the number of ring forms per trophozoite and schizont fell to 0.5, 0.2 and 0.4, respectively.

Incorporation of ³H-hypoxanthine during a 14-h pulse was also measured in synchronized cultures supplemented with ascitic fluid. In the absence of ascitic fluid, there was a low rate of incorporation from 16 to 30 h, increased incorporation from 40 to 54 h and the highest rate of incorporation from 64 to 78 h. The increase in incorporation paralleled the increase of parasitaemia. In cultures supplemented with Hb28 cl1 or Hb26 cl3 there was a low rate of incorporation from 16 to 30 h followed by a decrease in incorporation for Hb28 cl1 and a slight increase for Hb26 cl3 from 64 to 78 h. Here again, the rate of incorporation paralleled the level of parasitaemia (Fig. 1).

The IgG fraction of Hb26 cl3 ascitic fluid was shown to have full inhibitory activity (Table 2); the activity of Hb26 cl3 ascitic fluid was lost after depletion of IgG. Similarly, IgG fraction purified from Hb28 cl1 at a final concentration of 0.25 mg ml⁻¹ showed an inhibitory activity of 95%. In such cultures inhibited by IgG fractions, schizont degeneration was also present.

The amount of monoclonal antibodies needed to cause 50% inhibition of the culture growth after two multiplication cycles of

P. falciparum was 8 µg ml⁻¹ of culture medium for Hb28 cl1 (1/80 dilution of ascitic fluid) and 16 µg ml⁻¹ for Hb26 cl3. *P. falciparum*-infected cultures were also internally labelled with ³⁵S-methionine for 4 h and lysed by 0.5% Nonidet P40 (ref. 13). The lysate was used as antigen in double immunoprecipitation experiments using monoclonal antibodies (Hb26 cl3, Hb28 cl1 and Hb31 cl2)¹³. The washed immune precipitates were analysed on 10% SDS-polyacrylamide gel electrophoresis gels. By autoradiography it was shown that Hb28 cl1 and Hb31 cl2 monoclonal antibodies reacted with a polypeptide of molecular weight (MW) 41,000 whereas Hb26 cl3 reacted with two polypeptides of MWs 96,000 and 36,000. These polypeptides are probably present on the merozoite surface. This was indicated by results of the indirect fluorescence test when an unfixed suspension of *P. falciparum* was reacted with monoclonal antibodies. Intense surface staining was seen using monoclonal antibodies Hb26 cl3, Hb28 cl1 and Hb31 cl2. In contrast, no surface staining was observed with Hb12 cl4 or Hb9 cl1.

Previous investigators have shown that some sera from individuals living in areas endemic for malaria inhibit the growth of *P. falciparum* culture^{14–16}, but they have not identified the specificity of the 'protective' antibodies. In the present experiment, antigens recognized by monoclonal antibodies inhibiting the growth of *P. falciparum* have been characterized. Interestingly, monoclonal antibodies with inhibitory activity were obtained from the Hb28 cl1 hybrid after fusion of spleen cells from mice infected with *P. berghei*, and reacted by immunofluorescence with *P. berghei* (trophozoites and schizonts), suggesting the presence of a common antigenic determinant on the surface of *P. falciparum* and *P. berghei* merozoites. Several hypotheses can be raised to explain the inhibitory activity of the monoclonal immunoglobulin. First, these antibodies may prevent the merozoite invasion of RBCs by binding to the merozoite surface and interfering directly or indirectly with the parasite ligand-RBC receptor. Second, they may bind to parasite antigens at the surface of infected RBCs at the schizont stage and thus modify the intracellular parasites' metabolism. Third, because schizonts containing RBCs are known to have an increased permeability, one cannot exclude the possibility that some antibody molecules would reach intracellular parasites at the last stage of development.

These monoclonal antibodies provide new tools for the purification of potentially protective antigens of *P. falciparum*, although the large-scale production of *Plasmodium* antigens is still limited by technical problems. An alternative approach is to use DNA recombinant technology to produce defined malarial antigens. In this respect, the protective monoclonal antibodies may be useful for the identification of *Escherichia coli* colonies secreting putative protective antigens¹⁷.

This work was supported by WHO, the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases and the Swiss National Foundation (grant no. 3.890-0.79).

Received 2 May; accepted 21 November 1980.

1. 3rd Annual Report on Special Program for Research and Training in Tropical Diseases (WHO, Geneva, 1979).
2. Cohen, S., Butcher, G. A. & Mitchell, G. H. *Adv. exp. Med. Biol.* **93**, 89–112 (1977).
3. Reese, R. T., Trager, W., Jensen, J. B., Miller, D. A. & Tantravaki, R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5665–5669 (1978).
4. Siddiqui, W. A. *Science* **197**, 388–389 (1977).
5. Yoshida, N., Nussenzweig, R. S., Potocnjak, P., Nussenzweig, V. & Aikawa, M. *Science* **207**, 71–73 (1980).
6. Freeman, R. R., Trejdosiewicz, A. J. & Cross, G. A. M. *Nature* **284**, 366–368 (1980).
7. Trager, W. & Jensen, J. B. *Nature* **273**, 621–622 (1978); *Science* **193**, 673–675 (1976).
8. Lambros, C. & Vanderberg, J. P. *J. Parasit.* **65**, 418–420 (1979).
9. Reese, R. T., Langreth, S. G. & Trager, W. *Bull. Wild Hlth Org. Suppl.* **1**, 53–62 (1979).
10. June, C. H., Contreras, C. E., Perrin, L. H., Lambert, P. H. & Mischer, P. A. *J. Immun.* **121**, 2154–2160 (1979).
11. Galfre, G., Hove, S. C. & Milstein, C. *Nature* **266**, 550–552 (1977).
12. O'Neill, P. & Johnson, G. D. *J. clin. Path.* **23**, 185–189 (1970).
13. Wilson, R. J. M. & Phillips, R. S. *Nature* **263**, 132–134 (1976).
14. Perrin, L. H., Ramirez, E., Liu, E.-H. & Lambert, P. H. *Clin. exp. Immun.* **41**, 91–96 (1980).
15. Cohen, S., McGregor, I. A. & Carrington, S. P. *Nature* **192**, 733–737 (1961).
16. Phillips, R. S., Trigg, P. I., Scott-Finnigan, T. J. & Bartholomew, R. K. *Parasitology* **65**, 525 (1972).
17. Broome, S. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **75**, 2746–2749 (1978).

Respective roles of laminin and fibronectin in adhesion of human carcinoma and sarcoma cells

Israel Vlodavsky* & Denis Gospodarowicz†

* Department of Radiation and Clinical Oncology, Sharett Institute, Hadassah University Hospital, Jerusalem, Israel

† Cancer Research Institute and the Departments of Medicine and Ophthalmology, University of California, Medical Center, San Francisco, California 94143

In a previous study¹, Ewing's sarcoma cells and colon carcinoma cells² failed to attach to plastic and to dishes coated with various collagen types I, III and IV, but adhered rapidly to extracellular matrix (ECM) produced by cultured corneal endothelial cells. However, although carcinoma cells required ECM and did not adhere to fibronectin, the sarcoma cells adhered and flattened almost equally well on either substrate¹. Thus, different adhesive proteins may mediate the attachment of sarcoma- and carcinoma-derived cells to extracellular matrices, the most likely being fibronectin^{3,4} and laminin^{5,6}. Fibronectin stimulates the adhesion of fibroblasts, but not epidermal cells, to collagen type IV (ref. 7) and could mediate the attachment of sarcoma cells. Laminin is confined to the lamina lucida region of basement membranes⁶ and has been localized to cellular adhesion sites⁸. Studies of the attachment of epidermal cells *in vitro* (V. P. Terranova, personal communication) suggest that it is adhesive for epithelial cells, and so could have the same role for carcinoma cells as that played by fibronectin for sarcoma cells. Cultured vascular endothelial cells secrete fibronectin⁹⁻¹¹ and laminin¹² into both the ECM and the culture medium, and we report here that pre-exposure of plastic dishes to such conditioned medium induces the attachment and flattening of both human colon carcinoma and Ewing's sarcoma cells. While laminin mediates the attachment and spreading of the former fibronectin is responsible for the attachment and flattening of the latter.

Figure 1 shows the time-course of attachment of carcinoma cells to various substrata. Less than 30% of cells seeded on plastic or fibronectin-coated dishes became firmly attached and no flattening was observed, regardless of time in culture (up to 10 days) and whether or not serum (10%) was present. In contrast, 70 and 80% of the seeded cells firmly attached and flattened within the first hour of incubation on dishes pre-exposed to conditioned medium or coated with ECM, respectively (Figs 1, 2). This was observed regardless of whether or not carcinoma cells were seeded in the presence of serum (10%). The best results, in terms of adhesion and percentage of firmly attached and flattened cells, were obtained with medium from endothelial cell monolayers cultured for more than 8 days. Serum-free conditioned medium collected 5-7 days after the cells had reached confluence also promoted obvious cell attachment and flattening. Similar results were obtained with dishes exposed overnight for 2 h (Fig. 2) to the conditioned medium and seeded with either trypsinized single carcinoma cells (Fig. 2b, c) or cell aggregates (Fig. 2). In the latter case flattening and migration of peripheral cells were observed within 1 h and led to complete dispersion of aggregates 6-8 h after seeding (Fig. 2b). When similar tumour cell aggregates were seeded on ECM-coated dishes, flattening and migration were observed within 30 min (Fig. 2a). Pre-exposure of dishes to medium conditioned by human skin fibroblasts, vascular smooth muscle cells, or corneal endothelial cells (Fig. 3), or to fresh medium containing 10% serum supplemented or not with fibroblast growth factor (FGF), did not promote cell adhesion, flattening and migration. Studies of the production of laminin by vascular and corneal endothelial cells have shown that depend-

ing on the growth stage (sparse actively growing versus confluent resting cultures) the medium conditioned by corneal cells contains 20- to 100-fold less laminin than medium conditioned by vascular endothelial cells.

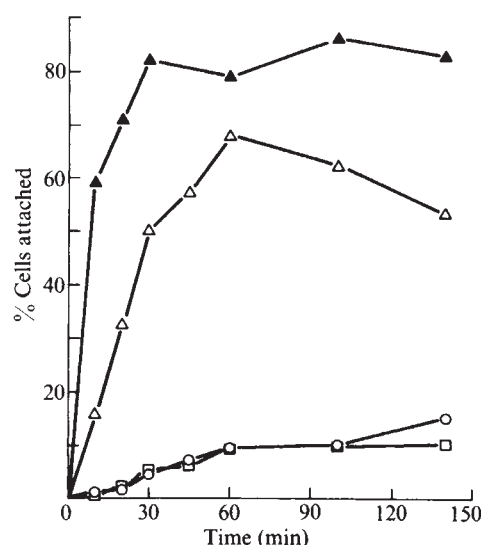


Fig. 1 Time-course of attachment of carcinoma cells to various substrates. Stock cultures of colon carcinoma cells grown on ECM-coated dishes were dissociated by treatment with STV (0.05% trypsin, 0.02% EDTA), and then 2×10^5 cells were seeded on plastic dishes (□), dishes coated with 10 µg each of affinity purified bovine plasma fibronectin (○, ref. 1), dishes coated with ECM (▲, ref. 1), or dishes coated with vascular endothelial cell conditioned medium (▼). To coat them with conditioned medium, dishes were exposed to a medium (DMEM, H-16 supplemented with 10% calf serum) taken from vascular endothelial cells maintained for 8 d in culture (from the day of seeding until 3-4 d after reaching confluence). This medium was centrifuged (1,500g for 5 min), incubated (14 h, 37 °C) on the dishes, and replaced with fresh conditioned medium before cells were seeded. Serum-free conditioned medium was collected from subconfluent endothelial cultures 7-9 d after they had been supplied with DMEM (H-16) containing no serum. Because the production and secretion of proteins by endothelial cells is greatly reduced when they reach confluence^{11,12}, medium conditioned by confluent cultures was less active than medium conditioned by actively growing vascular endothelial cells. At various times after seeding unattached carcinoma cells were removed by gentle pipetting and rinsing of the dishes (twice) with phosphate-buffered saline (PBS). The remaining firmly attached cells were dissociated with STV and duplicate cultures were counted with a Coulter counter. The variation in different determinations did not exceed $\pm 10\%$ of the mean.

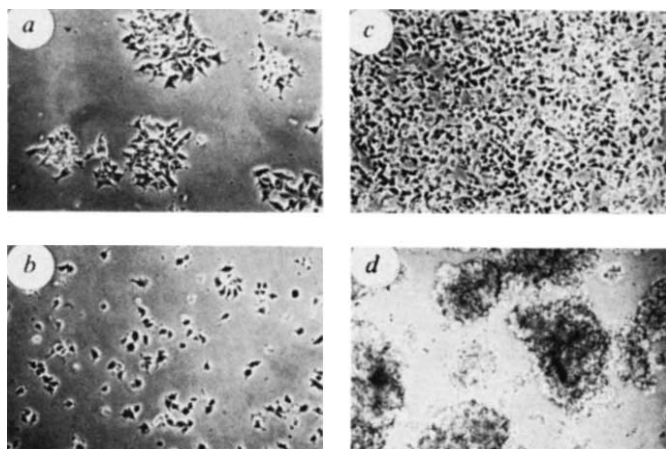


Fig. 2 Morphological appearance of carcinoma cells seeded on dishes pre-exposed to endothelial cell-conditioned medium. Floating cell aggregates (a, d) from stock cultures maintained on plastic or trypsinized single cells (b, c) from stock cultures maintained on ECM-coated dishes were seeded in DMEM containing 5% fetal calf serum on plastic (d) or on dishes pre-exposed (2 h, 37 °C) to endothelial conditioned medium as described in Fig. 1 legend (a, b, c). a, Cell spreading and migration from cell aggregates 8 h after seeding. b, Single cells 1 h after seeding. c, Confluent culture 6 d after seeding. The cells proliferate and form a layer of firmly attached and flattened cells.

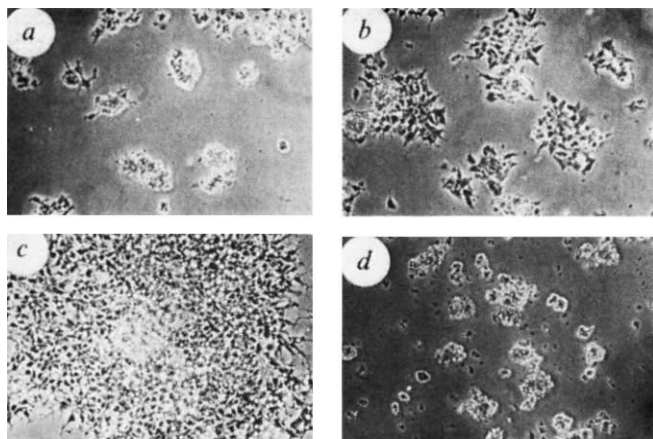


Fig. 3 Effect of conditioned media from various cell types on carcinoma cell attachment and spreading when cells are seeded and maintained in conditioned medium or seeded and maintained in fresh medium. Tissue culture dishes were pre-exposed (14 h, 37 °C) to media conditioned for 8 d by vascular endothelial cells (a–c) or corneal endothelial cells (d). Carcinoma cell aggregates taken without trypsinization from a culture maintained on plastic were seeded directly into the conditioned medium and observed 6 h later (a). Cell aggregates attached loosely to the substrate and only a small degree of cell flattening and migration was observed, even after 1 week in culture. When the conditioned medium was replaced with fresh medium, good cell attachment and spreading were observed after 2 h (b). This effect was then long-lasting (c, 1 week in culture). This demonstrates that, when carcinoma cells are seeded into conditioned medium-coated dishes, the conditioned medium has to be removed and replaced with fresh medium to promote cell attachment and spreading. Because poor cell attachment and flattening are observed when dishes contain conditioned medium (a), it is suggested that, although coating the plastic surface with conditioned medium induces cell flattening, soluble factor(s) which can adversely affect that process is also present in the conditioned medium. d shows cell aggregates 4 d after seeding in fresh medium into dishes coated with medium conditioned by corneal endothelial cells. Similar results were obtained with medium conditioned by vascular smooth muscle cells or human skin fibroblasts. In either case, and as was the case with uncoated tissue culture dishes, cell aggregates attached poorly and cell flattening was not observed.

Pre-exposure of dishes to the endothelial cell conditioned medium (either serum-free or containing fibronectin-depleted calf serum) also induced attachment of sarcoma cells. However, in this case and in contrast to carcinoma cells, coating of dishes

with purified fibronectin was either more or as effective in promoting cell attachment and subsequent flattening.

The nature of the agents mediating attachment and flattening was analysed by subjecting the conditioned medium to specific double immunoprecipitation with either rabbit anti-laminin⁶ or anti-fibronectin antisera⁹, followed by goat anti-rabbit IgG antiserum¹¹. Experiments with ³⁵S-methionine-labelled vascular endothelial cells had shown that these antisera, as determined by SDS-polyacrylamide gel electrophoresis, precipitate specifically and quantitatively the laminin¹² and fibronectin¹¹ present in endothelial cell conditioned medium. As Fig. 4b shows, carcinoma cells no longer became attached to dishes pre-exposed to conditioned medium if laminin had been removed by immunoprecipitation. On the other hand, immunoprecipitation of fibronectin (Fig. 4a) or collagen type IV (not shown) by specific antibodies had no effect on the attachment and flattening of carcinoma cells. In contrast, when the same immunoprecipitated fibronectin-free conditioned medium was used to coat dishes, sarcoma cells no longer became attached (Fig. 4d). The induced attachment and flattening of sarcoma cells were not affected by subjecting the conditioned medium to immunoprecipitation with anti-laminin (Fig. 4e), anti-collagen type IV or non-immune rabbit serum (Fig. 4f) followed by coating the dishes. These results show that the human carcinoma- and sarcoma-derived cells show a specific response to different adhesive glycoproteins, such as laminin and fibronectin, respectively.

Immunofluorescence has shown that the *in vivo* distribution of laminin is identical to that of collagen type IV (refs 6, 8) and so we compared the attachment of carcinoma cells to dishes coated with collagen types I, III, IV or V before being coated with conditioned medium. (Collagen would not promote cell attachment unless dishes were subsequently exposed to conditioned medium.) Collagen type IV gave the best results in terms of adhesion, percentage of firmly attached cells and prevention of retraction of flattened cells within days of initial attachment. Other collagens had either a small effect (type I) or were inhibitory (type II and in particular type V, Fig. 5d) compared with direct exposure to conditioned medium. When conditioned medium with a relatively low cell attachment-promoting activity was used (Fig. 5a), its effect was potentiated by precoating the dishes with collagen type IV (Fig. 5b). These results suggest that laminin may mediate the attachment of human carcinoma cells

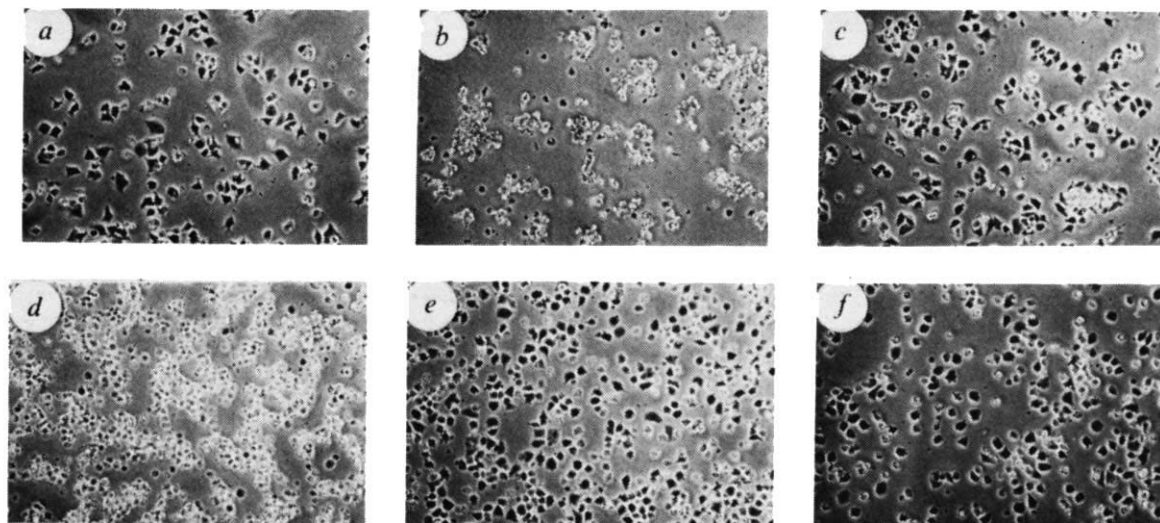


Fig. 4 Induction of tumour cell attachment promoted by coating dishes with vascular endothelial cell conditioned medium from which fibronectin or laminin had been removed by double immunoprecipitation. Vascular endothelial cell conditioned medium (1 ml) was preincubated (1 h, 37 °C) with 10 µl of rabbit antisera against fibronectin (a, d) or laminin (b, e), or with 10 µl of non-immune rabbit serum (c, f). Antibodies to laminin^{5,6} and bovine plasma fibronectin^{9,11} were the same as those used previously^{5,6,9,11}. The specificities of the antisera and lack of cross-reactivity between the various antigens have been established in these previous reports. The conditioned media were then incubated (2 h, 37 °C, followed by 14 h at 4 °C) with 30 µl of goat anti-rabbit IgG antiserum (35% ammonium sulphate cut), centrifuged (12,000g for 5 min) and the supernatant incubated in 35-mm tissue culture dishes for 12 h at 37 °C. The conditioned media were then removed and 2 ml of DMEM supplemented with 5% fibronectin-depleted calf serum and containing either 2×10^5 colon carcinoma cells (a–c) or Ewing's sarcoma cells (d–f) were then added to the dishes. Phase micrographs of cell attachment and spreading were taken 3 h after seeding. Conditioned medium pre-exposed to anti-collagen type IV (refs 5, 6) gave results similar to those observed after pre-incubation with non-immune rabbit serum. Similar results were obtained with serum-free vascular endothelial cell conditioned medium and with tumour cells seeded in DMEM containing 0.1% bovine serum albumin rather than 5% bovine serum.

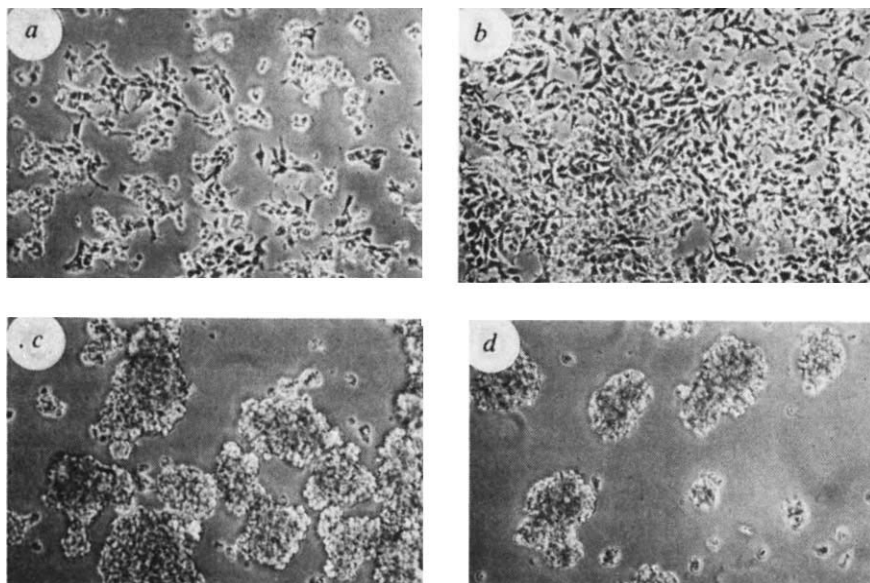


Fig. 5 Effect of precoating with types IV and V collagen on colon carcinoma cell attachment induced by a subsequent exposure to the vascular endothelial cell conditioned medium. Tissue culture dishes (35 mm) were coated with purified preparations of types IV (human placenta) or V (bovine cornea) collagen (10 μ g per dish) as previously described⁷. Plastic and collagen-coated dishes were then exposed (12 h, 37 °C) to conditioned medium taken 5 d after replacing the growth medium of subconfluent endothelial cultures. Carcinoma cells (10⁵ per dish) were seeded in fresh medium (DMEM, 10% FCS) and phase micrographs taken 3 d later. *a*, Pre-exposure to conditioned medium alone. *b*, Precoating with collagen type IV followed by exposure to conditioned medium. *c*, Dishes coated with collagen type IV alone. *d*, Precoating with collagen type V, followed by exposure to conditioned medium.

to type IV collagen which is characteristic of basement membranes.

Both laminin and fibronectin have been shown to be secreted underneath the endothelial cell layer and to be part of the ECM produced by these cells¹². In the ECM, they may function as conditioned medium does, and may promote the attachment and rapid spreading of carcinoma- and sarcoma-derived cells¹. The production of both laminin and fibronectin by vascular endothelial cells may facilitate the arrest of blood-borne metastatic cells¹³ that produce little or no such adhesive glycoproteins¹⁴. Metabolic labelling with ³⁵S-methionine of the Ewing's sarcoma and colon carcinoma cells (cultured on ECM) followed by specific immunoprecipitation had shown that these cells secrete no detectable laminin and small amounts of fibronectin (less than 3% and 0.1% of the newly synthesized proteins, respectively). Interaction of highly metastatic cells with the luminal surface of the vascular endothelium is followed by endothelial retraction and exposure of the underlying ECM, to which the tumour cells adhere strongly¹³, possibly through specific attachment-promoting molecules such as laminin and fibronectin. Cell attachment is then followed by migration, degradation and invasion of the tumour cells through the endothelial basement membrane^{13,15}. The presence of specific attachment factors in basement membranes, the ability or inability of cells to produce and/or interact with such factors, as well as the preferential interaction of these adhesive factors with a specific type of collagen may determine the localization of a given cell type to specific matrices⁷. Such specific cell-matrix interactions may direct the growth, migration and differentiation of normal cells^{16,19}, as well as the invasiveness and metastasis of tumour cells^{13,15}.

The colon carcinoma cell line (HS703T) was provided by Dr R. B. Owens, and the Ewing sarcoma cell line by the laboratory of J. E. Fogh (Sloan Kettering Institute). We thank Dr J. M. Foidart for affinity purified antibodies against laminin and collagen type IV, Dr S. Tseng for collagen types, G. M. Lui for technical assistance, Mr Harvey Scodel for assistance with the manuscript, and Professor Zvi Fuks for support and discussion. This work was supported by a grant from NIH to D.G.

Received 30 June; accepted 6 November 1980.

1. Vlodavsky, I., Lui, G. M. & Gospodarowicz, D. *Cell* **19**, 607-616 (1980).
2. Nelson-Rees, W. A., Hunter, L., Darlington, G. J. & O'Brien, S. J. *Cytogenet. Cell Genet.* (in the press).
3. Klebe, R. J. *Nature* **250**, 248-251 (1974).
4. Yamada, K. M. & Olden, K. *Nature* **275**, 179-184 (1978).
5. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J.-M. & Martin, G. R. *J. biol. Chem.* **254**, 9933-9937 (1979).
6. Foidart, J. M. *et al. Lab. Invest.* **42**, 336-342 (1980).
7. Murray, J. C., Stingl, G., Kleinman, H. K., Martin, G. R. & Katz, S. I. *J. Cell Biol.* **80**, 197-202 (1979).
8. Alitalo, K., Kurkinen, M., Vaheri, A., Krieg, T. & Timpl, R. *Cell* **19**, 1053-1062 (1980).

9. Birdwell, C. R., Gospodarowicz, D. & Nicolson, G. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3273-3277 (1978).
10. Jaffe, E. A. & Mosher, D. F. *J. exp. Med.* **147**, 1779-1791 (1978).
11. Vlodavsky, I., Johnson, L. K., Greenburg, G. & Gospodarowicz, D. *J. Cell Biol.* **83**, 468-486 (1979).
12. Gospodarowicz, D., Greenburg, G., Foidart, J.-M. & Savion, N. *J. Cell Physiol.* (in the press).
13. Kramer, R. H. & Nicolson, G. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5704-5708 (1979).
14. Smith, H. S., Riggs, J. L. & Mosesson, M. W. *Cancer Res.* **39**, 4138-4144 (1979).
15. Liotta, L. A., Tryggrason, K., Garbisa, S., Hart, I. & Folts, C. M. *Nature* **284**, 67-68 (1980).
16. Grobstein, C. *Cancer Inst. Monogr.* **26**, 279-299 (1967).
17. Wessels, N. K. in *Tissue Interactions and Development*, 213-229 (Benjamin, Menlo Park, California, 1977).
18. Gospodarowicz, D., Delgado, D. & Vlodavsky, I. *Proc. natn. Acad. Sci. U.S.A.* **77**, 4094-4098 (1980).
19. Gospodarowicz, D. & Ill, C. R. *Proc. natn. Acad. Sci. U.S.A.* **77**, 2726-2730 (1980).

cis-Interacting genes in the S region of the murine major histocompatibility complex

James Michaelson, Arturo Ferreira & Victor Nussenzweig

Irvington House Institute and Department of Pathology, New York University Medical Center, 550 First Avenue, New York, New York 10016

Insight into the control of gene expression may be gained by analysing genetic systems marked by both regulatory and structural variants. In such systems one can determine whether a regulatory element controls structural genes on both chromosomes or only on the chromosome to which it is linked. The latter may be detected in individuals heterozygous at both the regulatory and structural loci, in which case the effect of each regulatory allele is seen to be exerted only on the *cis*-located structural allele. In prokaryotic organisms, the identification of *cis* interaction of this sort has allowed elucidation of many features of genetic regulation, first for the *lac* operon¹ and subsequently for a variety of other systems². In higher organisms, however, there have been few opportunities to observe *cis*-interacting genes³⁻¹³. The most thoroughly characterized mammalian system in this regard is the murine β -glucuronidase locus described by Paigen and his colleagues¹⁴⁻¹⁶, in which *cis* interaction has been shown to occur between two closely linked genetic elements—the β -glucuronidase structural gene itself and an androgen-activated regulatory gene which controls the quantity of β -glucuronidase expressed. We report here that *cis*-interacting genetic elements are also found in the S region of the mouse major histocompatibility complex *H-2*.

Table 1 S-region γ -chain variants detected by IEF

	Haplotype	pI of γ chain
Ss(C4)	$H-2^{s,p,f,i,w17,w19}$	7.4
	$H-2^{d,k,r,u,q,b,w7}$	6.9
	$H-2^w$ (undesignated)	6.5
Slp	$H-2^{d,p,u,s,i}$	6.7
	$H-2^{w7}$	6.5

Results are summarized from refs 25–27.

The S region controls two structurally similar serum proteins^{17,18}, Ss (which has been shown to be C4 of the complement system^{19–22}) and Slp (sex-limited protein). Ss(C4) and Slp are each composed of three chains, α (molecular weight (MW) 100,000), β (MW 75,000) and γ (MW 35,000); in both cases the three subunits are derived from 200,000-MW precursors^{18,23}. Ss(C4) may be found in two phenotypes—high and low quantity in plasma (a 20-fold difference)—with the S region containing the genes determining phenotype^{17,24}. We have analysed the Ss(C4) and Slp proteins for biochemical variants which would serve as markers of the structural genes themselves. Recently, we described such variants (herein called *pI* variants)^{25–27}, identified by the isoelectric point of the γ chain (Table 1). The genetic determination of isoelectric point was found to map to the S region, where the previously described regulatory genes are located. Although the most straightforward explanation of the *pI* polymorphism is that of allelic variants of the structural genes, this has not been proved directly and other explanations are possible. Peptide mapping experiments to resolve this question are now underway. In any event, this need not hinder the formal argument of *cis* interaction between *pI* genes and the genes controlling expression.

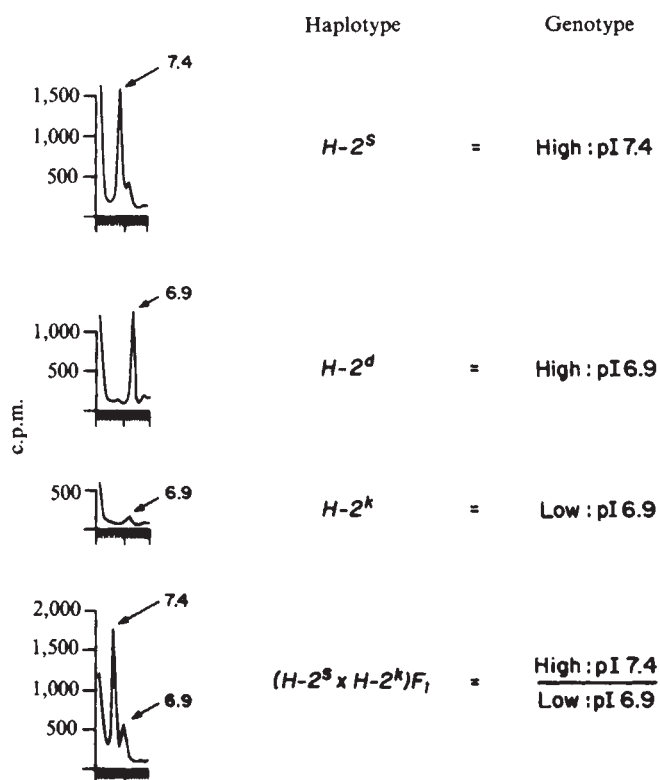


Fig. 1 Cylindrical IEF gels of the γ chain of Ss protein from $H-2^s$, $H-2^d$, $H-2^k$ and $(H-2^s \times H-2^k)F_1$ plasma. Ss precipitation was performed after removing Slp from plasma. Analogous results were obtained when Ss was analysed by two-dimensional separation (slab IEF, followed by SDS-polyacrylamide gel electrophoresis).

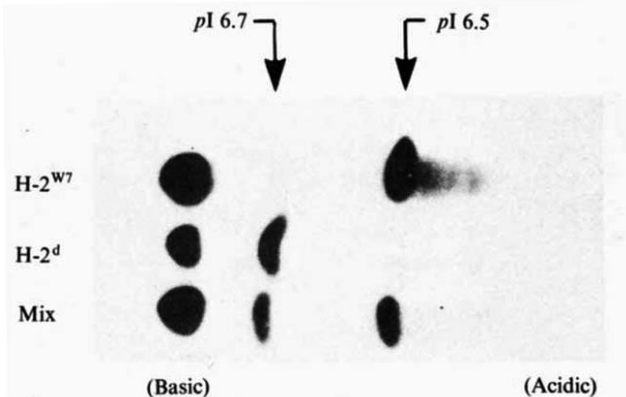


Fig. 2 Slab IEF (pH gradient 7.0–3.7) showing Slp γ chain of $H-2^{w7}$ (C3H.W7) and $H-2^d$ (DBA/2) mice, and a mixture of the two samples. The identification of the *pI* 6.7 and 6.5 spots as γ chains of MW 35,000 was made by applying individual strips to the top of an SDS-polyacrylamide gel, thus separating the components according to molecular weight (not shown) (see refs 25–27 for technical details).

In our analysis of Ss(C4) expression, we examined the interaction between two genetic elements: that determining the *pI* of the γ chain ($H-2^s = pI 7.4$, $H-2^k = pI 6.9$) and that determining the quantity of Ss(C4) ($H-2^s = \text{high}$, $H-2^k = \text{low}$). ($H-2^s \times H-2^k$) F_1 mice (high: 7.4/low: 6.9) were constructed. It was reasoned that, if the *regulatory* and *pI* genes interacted in *trans* (that is, through a diffusible control substance), equal quantities of 7.4 and 6.9 would be seen. If the interaction was *cis* only, however, a high quantity of 7.4 would be seen, with a low quantity of 6.9, and indeed this was what was found (Fig. 1).

We realize, however, that even though *cis* interaction is formally demonstrated, this might be due to differential labeling or breakdown of the two types of molecules. That the two genes are not precisely the same is seen by the fact that strains of both *Ss high : pI 7.4* and *Ss high : pI 6.9* type have been detected (Fig. 1). However, we do not know whether the S-region *regulatory* and *pI* genes are separate or overlapping entities (in terms of genetic fine structure).

We then performed the same type of analysis for the control of Slp expression. In most Slp^+ strains of mice (such as the $H-2^d$ strain described here), Slp is found in the sera of males but not females²⁸. This sex limited expression occurs because androgens are necessary to induce the Slp protein, as has been shown by Passmore and Shreffler²⁹ (castrated males are Slp^- , whereas females which are injected with testosterone propionate become Slp^+ (ref. 29)). Mice of an exceptional haplotype ($H-2^{w7}$), however, are Slp^+ in both males and females because expression is independent of androgen, as has been demonstrated by Hansen and Shreffler (such mice continue to produce Slp after castration, and indeed after being made genetically insensitive to testosterone by the *Tfm* mutation³⁰). Taken together, these observations identify a hormone-driven regulatory gene controlling the expression of Slp. It has two allelic forms: androgen dependent (AD) (that is, $H-2^d$) and androgen independent (AI) ($H-2^{w7}$). We have found that these two haplotypes also differ at their *pI* genes ($H-2^d = pI 6.7$, $H-2^{w7} = pI 6.5$)²⁷ (Fig. 2). We constructed ($H-2^d \times H-2^{w7}$) F_1 mice (AD: 6.7/AI: 6.5). If the interaction between the *regulatory* and the *pI* genes occurs by a *trans* mechanism, both males and females would be positive for both *pI* 6.7 and 6.5 Slp 's. If, however, interaction occurred in a strictly *cis* fashion, males would be positive for the *pI* 6.7 and 6.5 forms, but females (lacking androgens to turn on *cis*-located *pI* 6.7 product) would express only *pI* 6.5 Slp . Comparison of the isoelectric focusing (IEF) gels of Slp from males and females suggests that this is indeed the case (Fig. 3). Thus, for both Ss(C4) and Slp, the interaction of *regulatory* and *pI* alleles occurs only when they are located *cis*.

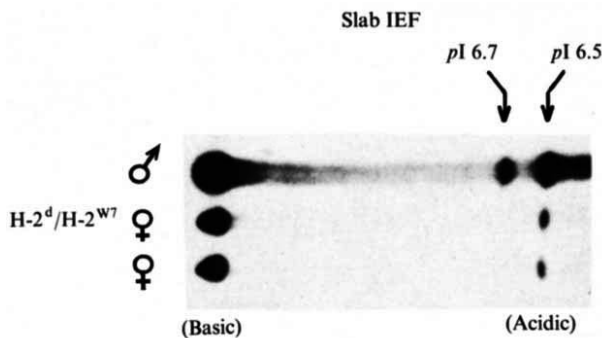


Fig. 3 Phenotype of the γ -chain SIp of (C3H.W7 $\times$ DBA/2) F_1 ($H-2^d/H-2^{w7}$) = (AD: pI 6.7)/(AI: pI 6.5), seen on slab IEF gels (pH gradient 7.7–4.0). (Components other than the γ chains are nonspecific contaminants.)

As seen in these experiments, the use of IEF to detect *S*-region structural polymorphism permits the simultaneous measurement of both allelic gene products in heterozygotes. From such data, inferences can be drawn about the properties of the genes which control the expression of the variants, as, for example, *cis* restriction of *S*-region regulatory genes. Further experiments of this sort should reveal in greater detail the properties of these regulatory genes. Two features of *S* region expression are noteworthy in this regard. First, because androgens control the serum levels of Ss(C4) (quantitatively)²⁴ and SIp (absolutely), the analysis of these two proteins may well elucidate some features of the nature of hormone regulation of gene activity. Second, Ss(C4) and SIp have been shown to be synthesized in macrophages and liver parenchymal cells, but not in a variety of other cell types³¹. Presumably, there are genetic elements which control this pattern of synthesis, but where they are or how they exert their effects is unknown. These regulatory elements would be identifiable by tissue-specific genetic polymorphism of Ss(C4) and SIp, if indeed such variation exists. The possibility of such polymorphism is suggested by the observation of Roos *et al.*¹⁸ that *in vitro* synthesis of SIp is seen in macrophages of $H-2^{w7}$ but not $H-2^s$ or $H-2^d$ genotype, although mice of all three types have the protein in their sera. The detailed analysis of this sort of polymorphism, including the use of IEF to test for *cis* and *trans* interaction with *pI* genes, should be informative both for the specific case of the *S* region and with respect to the general question of the control of gene expression during differentiation.

We thank Drs E. A. Boyse, L. Silver and D. Bennett for critical review of the manuscript, and Dr Yuri Bushkin for help with the IEF technique. We also thank Ms Holly Yudkowitz, Mr Victor La Regina and Mr Daniel Eichinger. This work was supported by NIH grants AI-13224, AI-00270 and CA-09149.

Received 23 June; accepted 16 October 1980.

- Jacob, F. & Monod, J. *J. molec. Biol.* **3**, 318–356 (1961).
- Miller, E. (ed.) *The Operon* (Cold Spring Harbor Laboratory, New York, 1979).
- Phillips, R. J. *Genetics* **51**, 485–495 (1966).
- Glekeler, W., Faversham, J. & Cohn, M. *J. exp. Med.* **148**, 1122 (1978).
- Dickinson, W. J. & Carson, H. L. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4559–4562 (1979).
- Dickinson, W. J. *Dev. Biol.* **42**, 131–140 (1975).
- Boubelik, M., Lengerova, A., Bailey, D. W. & Matousek, A. *Dev. Biol.* **47**, 206–214 (1975).
- McCaron, M. *et al. Genetics* **91**, 275–293 (1979).
- Daniel, W. L. *Biochem. Genet.* **14**, 1003–1018 (1976).
- Carlson, P. *Genet. Res.* **17**, 53–81 (1971).
- Jarry, B. & Faulk, D. *Molec. gen. Genet.* **135**, 113–122 (1974).
- Rawls, J. W. & Fristrom, F. W. *Nature* **255**, 738–740 (1975).
- Lewis, E. B. *Nature* **276**, 565–570 (1978).
- Paigen, K., Meisler, M., Felton, J. & Chapman, V. *Cell* **9**, 533–539 (1976).
- Swank, R. T. *et al. Recent Prog. Horm. Res.* **34**, 401–438 (1978).
- Paigen, K., Labarca, C. & Watson, G. *Science* **203**, 554–558 (1979).
- Shreffler, D. C. & Passmore, H. C. *Genetics* **48**, 9 (1963).
- Roos, M., Atkinson, J. P. & Shreffler, D. C. *J. Immun.* **121**, 1106–1115 (1979).
- Lachmann, P. J., Grennan, D. & Martin, A. *Nature* **258**, 242–243 (1975).
- Meo, J., Krasteff, T. & Shreffler, D. C. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4536–4560 (1975).
- Ferreira, A., Nussenzweig, V. & Gigli, I. *J. exp. Med.* **148**, 1186–1197 (1978).

- Curman, B. *et al. Nature* **258**, 243–245 (1975).
- Hall, R. E. & Colten, H. R. *Proc. natn. Acad. Sci. U.S.A.* **74**, 1707–1710 (1977).
- Hansen, T. H., Krasteff, T. N. & Shreffler, D. C. *Biochem. Genet.* **12**, 281–293 (1974).
- Ferreira, A., Michaelson, J. & Nussenzweig, V. *Z. Immun.* (in the press).
- Ferreira, A., Michaelson, J. & Nussenzweig, V. *J. Immun.* **125**, 1178–1182 (1980).
- Ferreira, A., Michaelson, J. & Nussenzweig, V. *Immunogenetics* (in the press).
- Passmore, H. C. & Shreffler, D. C. *Biochem. Genet.* **4**, 351–365 (1970).
- Passmore, H. C. & Shreffler, D. C. *Biochem. Genet.* **5**, 201–209 (1971).
- Hansen, T. H. & Shreffler, D. C. *J. Immun.* **117**, 1507–1513 (1976).
- Saunders, D. & Edidin, M. *J. Immun.* **112**, 2210–2218 (1974).

Detection of CML determinants associated with $H-2$ controlled E_β and E_α chains

Antonio Juretić*†, Zoltan A. Nagy* & Jan Klein*

* Max-Planck-Institut für Biologie, Abteilung Immunogenetik, Tübingen, FRG

† Department of Physiology, Faculty of Medicine, University of Zagreb, Zagreb, Yugoslavia

The serologically detectable molecules encoded by the $H-2$ complex, the major histocompatibility complex (MHC) of the mouse, fall into two classes—class I and class II (ref. 1). The class I molecules encoded by the *K* and *D* loci have a molecular weight of 44,000 and are noncovalently associated with β_2 microglobulin which is not controlled by the MHC. The class II molecules are of two kinds, A and E, each consisting of two noncovalently associated polypeptide chains, α (M_r ~34,000) and β (M_r ~28,000)^{2,3}. Three of the four chains, A_α , A_β and E_β , are controlled by loci in the *I-A* subregion, whereas the locus controlling the E_α chain is located in the *I-E* subregion of the $H-2$ complex. Thus the loci coding for the E_α and E_β chains are separated by at least one (*J*) and perhaps more loci^{3,4}. It has been shown that the E_α and E_β chains are synthesized independently, and that the E_α chain is required for the insertion of the E_β chain in the plasma membrane⁴. We demonstrate here that the $E_\alpha E_\beta$ complex (the E molecule) can evoke *in vitro* cell-mediated lymphocytotoxicity (CML) without previous sensitization *in vivo*, and that the strong CML reactivity is directed against allele-specific determinants on the highly polymorphic E_β chain.

To generate CML against the E molecules, we used three strain combinations selected from the $H-2$ recombinant pool such that the responders differed genetically from the stimulators only in the region between the *A* and *S* loci (Table 1). The region may contain, in addition to the loci controlling E_α and E_β chains, the *B*, *J*, *C* and perhaps other, as yet unidentified loci. Four lines of evidence support the conclusion that the CML is directed against E rather than against other molecules encoded by the *A-S* interval. (1) When tested on a panel of $H-2$ recombinant strains, reactivity of some of the effector cells with some of the strains excludes the *B*, *J* and *C* loci from coding for the target-cell determinants. (2) The same panel test indicates that the strain distribution pattern of the determinants detected by the effector cells correlates with the distribution pattern of alleles at E_α - and E_β -encoding loci. (3) The CML typing data fit well with what is known about the expression of E_α - and E_β -encoding loci^{3,4}. (4) Cytotoxicity generated across disparities from the *I-B* to *S* region is specifically blocked by a monoclonal antibody against the Ia.7 determinant on the E_α chain⁵.

The CML was generated by incubating unprimed spleen cells of the responder strain in a standard mixed-lymphocyte culture (MLC) with γ -irradiated spleen cells of the stimulator strain for 5 days. After incubation, the effector cells were tested on a panel of lipopolysaccharide (LPS)-stimulated, ⁵¹Cr-labelled target cells and specific release of radioactivity was taken as a measure of cell lysis. In all three strain combinations, reproducibly high

Table 1 Panel testing of primary CML generated against $E_\beta E_\alpha$ molecules

Haplotypes	Target cell $H-2$ Regions				% Net ^{51}Cr release by effectors		
	K	Regions		D	B10.S(7R) α B10.S(9R) (anti- E_β^7)	B10.GD α B10. D2(R101) (anti- E_β^7)	B10.A(4R) α B10.A(2R) (anti- E_β^7)
		I-A ($A_\alpha A_\beta$)	I-E (E_β)				
k	k	k	7	k	19(16-21) ³	12	37(25-45) ³
a, a1	k	k	7	d	19(12-22) ³	12	41(34-52) ³
h1, h2, h3	k	k	7	b	16(7-23) ⁹	8(4-10) ⁴	38(28-47) ¹⁵
m, m1	k	k	7	q	18(16-19) ²	10	39
aq1	k	k	7	f	17(10-28) ³	11	36(27-44) ²
y1	q	k	7	d	19(7-25) ⁴	8	40(34-44) ³
by1	q	k	7	b	17(15-20) ³	NT	30(27-33) ²
t1	s	k	7	d	14(6-22) ⁵	9(6-12) ²	33(24-43) ³
at4	s	k	7	b	15	NT	39(38-40) ²
an1	s	k	7	f	14(8-17) ³	NT	40(35-45) ²
as1	k	k	(k)	0	0(-3-6) ⁵	4(3-5) ²	3(-2-7) ⁴
h4	k	k	(k)	0	0(-7-7) ⁵	3(0-6) ²	0 ⁷
i3, i5	b	b	b	7	16(10-20) ⁶	8(6-11) ³	31(21-44) ⁶
i7	b	b	(b)	0	2(-3-6) ²	4	11(7-14) ³
b	b	b	(b)	0	0(-4-5) ³	1(-2-3) ²	8(5-11) ⁴
g1, g3	d	d	d	7	11(8-14) ²	21(16-26) ⁶	19(16-22) ²
o1	d	d	d	7	6(5-6) ²	15(12-16) ³	16(15-16) ²
d	d	d	d	7	6(1-13) ⁴	20(16-24) ²	17(15-18) ²
g2	d	d	(d)	0	-2(-6-2) ³	0 ⁶	2(-3-6) ⁴
t3, t4	s	s	s	7	33(24-38) ¹¹	11(10-12) ²	19(11-34) ¹⁶
t2	s	s	(s)	0	0 ¹¹	5(4-5) ³	5(-1-12) ⁷
s	s	s	(s)	0	1(-3-5) ³	3 ²	3(1-6) ³

CML were set up with 10×10^6 responder and 8×10^6 irradiated stimulator spleen cells in 4.5 ml of Alpha minimal essential medium (Gibco) containing 10% fetal calf serum, and 3×10^{-5} M 2-mercaptoethanol. After 5 days in culture, the cells were tested for lysis of ^{51}Cr -labelled LPS-blasts at different effector:target (E:T) ratios. For designation of target-cell haplotypes see ref. 8. $H-2$ regions coding for potential CML determinants are shown. Alleles of the E_β locus in brackets mean failure of cell-surface expression of the relevant polypeptides. Only plateau levels of killing, obtained at E:T 100:1 to 160:1, are shown. At high E:T ratios, the % net release, that is, the difference between % specific killing of various targets and % specific killing of responder haplotype targets, showed little variation between individual experiments, and was characteristic of the CML combination tested. (Calculation of % specific release was as in Fig. 1 legend.) This fact allowed pooling of data from several experiments. The data are expressed as mean and range of % net release obtained in 2-16 experiments. Superscript numbers show the number of individual experiments (not shown where target haplotypes were tested once).

specific lysis was observed. However, in each combination the reaction was unidirectional—reaction was obtained in the direction B10.A(4R) anti-B10.A(2R) (hereafter 4R α 2R), B10.S(7R) anti-B10.S(9R) (7R α 9R) and B10.GD anti-B10.D2(R101) (GD α R101); all attempts to generate effector cells in the opposite direction have failed. This unidirectional response agrees well with present knowledge concerning the expression of E molecules in the plasma membrane. Jones *et al.*⁴ demonstrated that $H-2$ haplotypes b, q, s and perhaps also f failed to produce functional E_α chains and that this failure prevents the E_β chains from being inserted into the plasma membrane. Thus cells of these four haplotypes bear no E molecules on their surface. In the strain combinations used, one of the strains in each pair always expresses E molecules on the cell surface and the other does not, and we obtained CML only when the responder was the nonexpressor and the stimulator the expressor—never in the opposite direction. In the combination 4R α 2R, unidirectional response was previously observed in MLC, and a complicated explanation involving unequal crossing-over and gene deletion was proposed⁶. Such explanations are no longer necessary; the nonexpression of the E molecule in the membrane fully explains the data—the β -encoding gene cannot be deleted in the B10.A(4R) strain because the E_β chain is present in the cytoplasm of this nonexpressor.

When tested on a panel of strains carrying independent and recombinant $H-2$ haplotypes (Table 1), the three effector-cell types gave two kinds of reactivity, one that was comparable in strength with that obtained against the stimulator strain, and one that yielded approximately half as much specific ^{51}Cr release as the lysis of stimulator-type targets (Fig. 1). We shall refer to these two types of response as 'strong' and 'intermediate', respectively. The distinction between the strong and intermediate reactions was also confirmed by cold-target inhibition studies (example shown in Fig. 1).

Table 1 also shows the distribution of alleles at the E_α - and E_β -encoding loci among the tested strains. The E_α locus is shown to possess only two alleles, one designated '7' because it

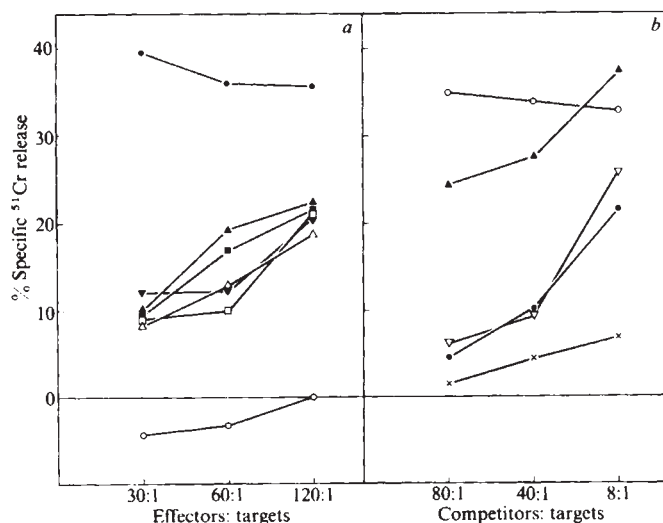


Fig. 1 Reactivity patterns of B10.S(7R) anti-B10.S(9R) effector cells. *a*, Direct cytotoxicity; *b*, cold-target inhibition of cytotoxicity against B10.S(9R) targets. Targets or competitors: $\blacktriangle$, t1 (A.TL); $\circ$, t2 (B10.S(7R)); ∇ , t3 (B10.HTT); $\bullet$, t4 (B10.S(9R)); $\triangle$, i3 (B10.A(3R)); $\square$, a(A/J); $\blacksquare$, a(B10.A); $\blacktriangledown$, a(A.Tla^b); $\times$, both targets and competitors are t2. Per cent ^{51}Cr -release was computed using the formula:

$$\frac{\text{c.p.m. supernatant} \times 2}{2 \times \text{c.p.m. cell lysate} + \text{c.p.m. supernatant}} \times 100$$

where the denominator represents total counts per well, when the sample size is half-volume supernatant and half-volume zaponin-lytate. Per cent specific ^{51}Cr release was calculated by subtracting per cent release in control groups from that in experimental groups. The controls consisted of labelled target cells mixed with normal spleen cells at ratios corresponding to E:T ratios in the experimental groups. Spontaneous release (control groups) ranged from 17 to 35%, depending on the target strain.

codes for the serologically detectable antigen Ia.7 (ref. 7) and another designated '0' because it codes for the absence of Ia.7 in the plasma membrane (however, in reality, there may be only one allele at the E_α -encoding locus and the expression as opposed to nonexpression of the E_α chain in the membrane may be controlled by another locus). The distribution of the 7 allele is that determined by biochemical methods and serological typing for Ia.7 (refs 7, 8).

The E_β -encoding locus is shown to possess at least as many different alleles as there are independent $H-2$ haplotypes. This conclusion is based on biochemical data which demonstrate that the E_β chains controlled by independent $H-2$ haplotypes differ in their peptide maps³ (the E_β alleles b , s and k have not yet been distinguished serologically, probably because correct strain combinations for the production of the relevant antibodies have not been used).

The CML combinations 7R α 9R and GD α R101 gave strong CML reactivity only against target cells carrying the 7 allele at the E_α -encoding locus and, at the E_β -encoding locus, the same allele as the stimulator cell (Table 1). As the E_α chains of most Ia.7-positive strains tested derive from H-2^k, the data strongly indicate that these two types of effector cell recognize allele-specific (private) determinants on the E_β chain. The third combination 4R α 2R gave strong CML against target cells carrying the k and b , but not the d and s alleles at the E_β -encoding locus, together with the 7 allele at the E_α -encoding locus. The panel test in Table 1 also demonstrates that recognition of the E_β determinants is not restricted by class I antigens, as target cells that share the E molecule with the stimulator cells, but differ from them at the K and D region, are lysed with equal efficiency. We conclude that the allelic forms of the E_β chain are not only chemically distinct, but also differ functionally, in terms of recognition by cytotoxic T lymphocytes.

The intermediate CML reactivities in all three combinations depend on the presence of E molecules in the target cells' plasma membrane, and thus correlate with the expression of Ia.7 (E_α chain) by the cells (Table 1). Our most recent results suggest that the determinants detected by intermediate CML reactivity are also found on the E_β chain. So far, direct contribution of the E_α chain to the formation of CML determinants could not be demonstrated⁵. Target cells which carry the 0 allele at the E_α -encoding locus, and thus do not express the E molecule on the membrane, are not lysed. The marginal killing of b and $i7$ target cells by 4R α 2R effectors is an apparent exception; however, the absence of this reactivity on g2 target cells maps the determinant(s) recognized to the K or $I-A$ region.

We have also obtained CML reactivity against the E molecule in the strain combination B10.D2(R107) anti-B10.A(3R). This CML combination involves multiple genetic disparities at the E and C regions, the Tla locus and possibly also at other loci to the right of the S region^{8,9}. This effector-cell type seemed to preferentially recognize target cells of the $i3$ and $i5$ haplotypes, which express the b allele at the E_β -encoding locus together with the 7 allele at the E_α -encoding locus. In addition, they exhibited cross-reactivities independent of the recognition of the E molecule. The genetic basis for these cross-reactions is being investigated.

In conclusion, our results, together with other data¹⁰, demonstrate that the two gene-controlled E molecule serves as a target antigen for recognition by cytolytic T lymphocytes. The CML determinants seem to be localized primarily, if not exclusively, on the highly polymorphic E_β chain. The implications of these findings with regard to the function of the E molecule in immune responses will be discussed in detail elsewhere¹¹. But as the E_β and E_α chains also bear serologically detectable determinants¹², participate in Ir-gene control of the immune response and immune suppression¹³, and stimulate mixed-lymphocyte reaction¹⁴, there is obviously no need to postulate separate loci for all these functions.

This work was supported in part by grant Wa 139/10/AI.5 from the Deutsche Forschungsgemeinschaft. We thank Ms Gudrun Labib for technical assistance.

Received 25 July; accepted 21 November 1980.

1. Klein, J. *Science* **203**, 516–521 (1979).
2. Cullen, S. E., Freed, J. H. & Nathenson, S. G. *Transplant Rev.* **30**, 236–239 (1976).
3. Uhr, J. W., Capra, J. D., Vitetta, E. S. & Cook, R. G. *Science* **206**, 292–297 (1979).
4. Jones, P. P., Murphy, D. B. & McDevitt, H. O. *J. exp. Med.* **148**, 925–939 (1978).
5. Juretić, A. *et al. Immunogenetics* (submitted).
6. Lozner, E. C., Sachs, D. H., Shearer, G. M. & Terry, W. D. *Science* **183**, 757–759 (1974).
7. David, C. S. & Shreffler, D. C. *Transplantation* **18**, 313–321 (1974).
8. Klein, J., Flaherty, L., VandeBerg, J. L. & Shreffler, D. C. *Immunogenetics* **6**, 489–512 (1978).
9. Egorov, J. K., Apt, A. S. & Vedernikov, A. A. *Immunogenetics* **5**, 285–290 (1977).
10. Fischer Lindahl, K. & Hausmann, B. *Immunogenetics* (in the press).
11. Klein, J. *et al. Nature* (submitted).
12. Lafuse, W. P., McCormick, J. E. & David, C. S. *J. exp. Med.* **151**, 709–715 (1980).
13. Benecerraf, B. & Germain, R. N. *Immun. Rev.* **38**, 70–119 (1978).
14. Peck, B. A., Klein, J. & Wigzell, H. *J. Immun.* **125**, 1078–1086 (1980).

Nonenzymatic hydroxylations of proline and lysine by reduced oxygen derivatives

Robert L. Trelstad, Karen R. Lawley & Lewis B. Holmes

Experimental Pathology and Embryology Laboratories, Shriners Burns Institute, Departments of Pathology and Pediatrics, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02114

The biosynthesis of *trans*-3- and *trans*-4-hydroxyprolines and 5-hydroxylysine in animal cells requires polypeptide proline or lysine, enzymes and cofactors including iron, and possibly involves peroxidatic intermediates¹. Several laboratories have reported the presence of low-molecular-weight hydroxyproline and hydroxylysine peptides in cell and organ cultures^{2–5}. We found that these small peptides contained the *trans*-3 and *cis*-4 isomers of hydroxyproline as well as *trans*-4 ones and that their production was not completely inhibited by α,α -dipyridyl, an iron chelator and effective inhibitor of enzyme-mediated hydroxylations⁵. It is known that oxygen or hydrogen peroxide in the presence of metal can hydroxylate proline and other aromatic compounds^{6–11}. We show here that reduced oxygen derivatives can hydroxylate both free and polypeptide-bound proline and lysine, and that scavengers of hydroxyl radicals suppress, but do not completely inhibit, this reaction. Reduced oxygen derivatives can be generated in normal and pathological circumstances¹², and some of the low-molecular-weight hydroxyproline and hydroxylysine peptides found in cell and organ cultures might be derived from these derivatives and therefore do not reflect collagen turnover, but rather some other cellular activity.

Free proline and lysine and polypeptide-bound forms of each, as well as three isomers of hydroxyproline, were exposed to several different reaction conditions (listed in Table 1), which contained or could generate superoxide anion, hydrogen peroxide or hydroxyl-free radicals¹².

The reaction products were identified using the following techniques: high-voltage electrophoresis¹³, thin-layer chromatography¹⁴, chemical derivatization and partitioning by solvent extraction followed by chromatophore generation (including characterization of the spectral absorption pattern of standards and the derivatives)¹⁵, nitrous acid resistance¹⁶, and behaviour on the amino acid analyser, including the reaction product absorption ratios at 440 and 570 nm and co-chromatography with known standards in at least two different elution conditions (Fig. 1).

The products of the reactions involving proline include at least four different hydroxyproline isomers—*trans*-3-hydroxy-

Table 1 Nonenzymatic hydroxylations of proline and lysine in free and polypeptide forms

Substrate	Reaction conditions	Reaction products*				
		<i>trans</i> -3-hyp	<i>trans</i> -4-hyp	<i>cis</i> -3-hyp	<i>cis</i> -4-hyp	5-hyllys
<i>Free forms:</i>						
L-proline	Standard†	2.7	2.6	4.2	3.2	5.4
	Standard except at 20 °C	1.9	1.8	2.1	1.5	4.3
	Standard except at 0 °C	1.5	1.3	1.9	1.3	4.1
L-lysine	Standard except Fe ²⁺ replaced at same concentration by Zn ²⁺	2.0	2.2	4.6	3.0	1.9
	Cu ²⁺		(trans-3 + unidentified)			2.6
	Hg ²⁺ , Ca ²⁺ , Mn ²⁺ , Mg ²⁺ , Pb ²⁺	‡	‡	‡	‡	‡
	Standard except proline increased fourfold	2.3	3.1	6.4	3.3	
	Standard except Fe ²⁺ increased 10-fold	7.3	15.3	22.8	30.6	31.0
	Standard with the addition of free radical scavengers:					
	50 µM mannitol	2.4	1.9	2.9	1.8	1.1
	50 µM sodium formate	1.9	1.6	2.5	1.5	3.0
	5 µM thiourea	2.2	2.1	3.0	2.2	0.1
	10 µM sodium benzoate	1.9	1.5	2.8	1.6	TR
	50 µM potassium chloride	3.1	1.6	4.1	3.3	2.7
	50 µM dimethyl sulphoxide	1.3	1.1	1.6	0.4	TR
	100 µM dimethyl sulphoxide	0.4	0.4	0.4	0.2	TR
	Standard with the addition of iron chelators:					
	10 µM α,α-dipyridyl	3.8	3.0	5.5	3.3	
	300 µM diethylenetriamine penta-acetic acid	3.4	3.2	5.2	3.4	0.6
Standard without H ₂ O ₂ and with hypoxanthine/xanthine oxidase:						
	with no other additions	2.3	3.5	4.9	5.2	TR
	with added catalase or superoxide dismutase	0.08	0.10	0.2	0.3	0.0
		0.05	0.06	0.3	0.3	0.0
<i>cis</i> -4-hydro	Standard		6.0		100.0	
<i>trans</i> -4-hydro	Standard		100.0		TR	
<i>trans</i> -3-hydro	Standard		(? 3,4-dihydroxyprolines)			
<i>Polypeptide forms:</i>						
Pro-Gly	Standard	10.5	42.0	20.8	85.6	
Pro-Gly + HNO ₂	Standard	2.0	43.4	TR	95.0	
Gly-Pro	Standard	5.7	139.7	TR	TR	
Gly-Pro-Ala	Standard	2.3	2.5	0.7	1.3	
Lys-Lys	Standard					38.7
Lys-Gly	Standard					21.6
Albumen	Standard	TR	9.6	—	—	27.4

TR, trace. *trans*-3-hyp, *trans*-3-hydroxyproline; *trans*-4-hyp, *trans*-4-hydroxyproline; *cis*-3-hyp, *cis*-3-hydroxyproline; *cis*-4-hyp, *cis*-4-hydroxyproline; 5-hyllys, 5-hydroxylysine.

* Data were determined by amino acid analyses without hydrolysis (free forms) or after hydrolysis in 6 M HCl (polypeptide forms). The presence of the metal ions did not interfere with the analyses. The values represent the moles of product formed per mole of proline or lysine (or *cis*-4 or *trans*-4) remaining at the end of the reaction ×100. Overall recovery of proline/lysine was less than 10%. The values represent averages of two to six determinations.

† Standard conditions were: 20 µM substrate, 0.2 mM ascorbate, 10 µM FeSO₄·7H₂O, 1.0 mM H₂O₂, all in 0.1 M NaH₂PO₄, pH 4.4, at 37 °C for 1 h, followed by reduction with 1 mM NaBH₄ at pH 8.0.

‡ These metals with proline gave no reaction; with lysine, 50% of Fe²⁺.

proline, *trans*-4-hydroxyproline, *cis*-3-hydroxyproline and *cis*-4-hydroxyproline. No differences were noted when D- or L-proline was used in free form. When free proline was the substrate, the relative amounts of each of these isomers produced were nearly equal in the conditions used for most of the experiments. However, with peptide-linked proline, there were significant changes in the distribution of the isomers produced. For example, free proline yields approximately equal amounts of *trans*-3-hydroxyproline and *trans*-4-hydroxyproline, whereas peptide-bound proline is predominantly converted to *trans*-4-hydroxyproline (Table 1). The proline sequence in peptide linkage also affected its relative reactivity and, interestingly, the configuration which is normally hydroxylated in collagen—Pro-Gly—was approximately five times more hydroxylated on a molar basis of starting substrate concentration than Gly-Pro or Gly-Pro-Ala. The proline or lysine recovered at the end of the reaction was less than 10% of the starting material. In addition to the products listed in Table 1, other products were noted and of particular interest was a substance which eluted from the amino acid analyser 7 min

before *trans*-4-hydroxyproline, which we have tentatively identified as 4-ketoproline¹⁷. This material was separated from *trans*-4-hydroxyproline on the amino acid analyser by adjustment of the pH of the first buffer to 2.68 (Fig. 1). Several other products, similar in chromatographic properties on the amino acid analyser to the several isomers of the 3,4-dihydroxyprolines, were detected, but not studied further^{17,18}.

The reaction products generated with free lysine as substrate included 5-hydroxylysine and a second substance which eluted 10 min later, which has not been identified (Fig. 1). With lysine as substrate in polypeptide form, the major product was 5-hydroxylysine.

We suspect that 3- and 4-ketoproline are intermediates in the reaction. The 3-keto form apparently reduces readily in the presence of ascorbate as *trans*-3-hydroxyproline consistently appeared without the addition of sodium borohydride. The 4-keto form was variably present in the initial reaction conditions, but could be quantitatively eliminated by the addition of the sodium borohydride. The 4-keto intermediate was also noted when free *cis*-4-hydroxyproline was used as substrate.

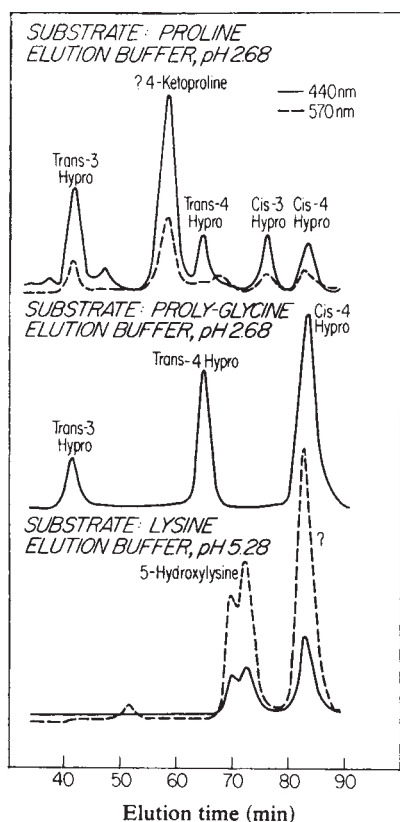


Fig. 1 Elution patterns of reaction products on the amino acid analyser of non-enzymatic hydroxylations of free proline (upper tracing), polypeptide-bound proline which was hydrolysed to free amino acids and then treated with nitrous acid (middle tracing) and free lysine (lower tracing). The solid tracing is the absorbance at 440 nm, the dotted tracing that at 570 nm. The pH 2.68 buffer was 0.18 M in Na^+ , the pH 5.28 buffer was 0.35 M in Na^+ . The identification of the eluting material by means other than co-chromatography with standards is detailed in the text. The two peaks with ? have not been characterized, but that with proline is probably 4-ketoproline.

cis-4-hydroxyproline, may be true biosynthetic products. Because *cis*-4-L-hydroxyproline is an analogue of L-proline and effective inhibitor of stable collagen triple-helix formation²⁸, its production *in vivo* would provide a novel mechanism for regulating net collagen production and add to the growing list of biological effects of oxygen radicals²⁹.

This work was supported by NIH grants HL 18714, HL 23591, GM 21700 and HD 09689, The March of Dimes Birth Defects Foundation and the Shriners Burns Institute. We thank Mary G. Kirby for technical assistance and Dr Charles Cintron for help with the thin-layer chromatography and high-voltage electrophoresis.

Received 30 April; accepted 16 October 1980.

1. Myllylä, R., Schubotz, L. M., Weser, U. & Kivirikko, K. *Biochem. biophys. Res. Commun.* **89**, 98–102 (1979).
2. Steinberg, J. *J. Cell Sci.* **12**, 217–234 (1973).
3. Nourse, P. N., Nourse, L. D. & Botes, H. S. *Afr. J. Sci.* **70**, 231–234 (1974).
4. Bienkowski, R. S., Cowan, M. J., McDonald, J. A. & Crystal, R. G. *J. biol. Chem.* **253**, 4356–4363 (1978).
5. Holmes, L. B. & Trelstad, R. L. *Dev. Biol.* **72**, 41–49 (1979).
6. Chvapil, M. & Hurych, J. *Nature* **184**, 1145 (1959).
7. Lampert, D. T. A. *Nature* **202**, 293–294 (1964).
8. Yip, C. C. *Biochim. biophys. Acta* **92**, 395–396 (1964).
9. Gruber, H. A. & Mellon, E. F. *Analyt. Biochem.* **66**, 78–86 (1975).
10. Udenfriend, S., Clark, C. T., Axelrod, J. & Brodie, B. B. *J. biol. Chem.* **208**, 731–739 (1954).
11. Breslow, R. & Lukens, L. N. *J. biol. Chem.* **235**, 292–296 (1960).
12. Hayaishi, O. & Asada, K. (eds) *Biochemical and Medical Aspects of Active Oxygen* (University Park Press, Baltimore, 1977).
13. Cintron, C., Peczon, B. D. & Kublin, C. L. *Analyt. Biochem.* **87**, 622–630 (1978).
14. Villanueva, V. R. & Lederer, E. *FEBS Lett.* **52**, 308–311 (1975).
15. Kivirikko, K. I. *Int. Rev. Connective Tissue Res.* **5**, 93–163 (1970).
16. Dziewiatkowski, D. D., Hascall, V. C. & Riolo, R. L. *Analyt. Biochem.* **49**, 550–558 (1972).
17. Mauger, A. B. & Witkop, B. *Chem. Rev.* **66**, 47–86 (1966).
18. Irreverre, F. & Witkop, B. *J. Chromat.* **43**, 127–128 (1969).
19. Michelson, A. M., McCord, J. M. & Fridovich, I. (eds) *Superoxide and Superoxide Dismutases* (Academic, London, 1977).
20. Foerder, C. A. & Shapiro, B. M. *Proc. natn. Acad. Sci. U.S.A.* **74**, 4214–4218 (1977).
21. DeVore, D. P. & Gruebel, R. J. *Biochem. biophys. Res. Commun.* **80**, 993–999 (1978).
22. Waykole, P. & Heidemann, E. *Connective Tissue Res.* **4**, 219–222 (1976).
23. LaBella, F., Keeley, F., Vivian, S. & Thornhill, D. *Biochem. biophys. Res. Commun.* **26**, 748–753 (1967).
24. Andersen, S. D. *Biochim. biophys. Acta* **93**, 213–215 (1964).
25. McCord, J. M. *Science* **185**, 529–531 (1974).
26. Petrone, W. F., English, D. K., Wong, K. & McCord, J. M. *Proc. natn. Acad. Sci. U.S.A.* **77**, 1159–1163 (1980).
27. Postlethwaite, A. E., Seyer, J. M. & Kang, A. H. *Proc. natn. Acad. Sci. U.S.A.* **75**, 871–875 (1978).
28. Rosenbloom, J. & Prockop, D. J. *J. biol. Chem.* **245**, 3361–3368 (1970).
29. Fridovich, I. *Science* **201**, 875–880 (1978).

Biosynthetic labelling of platelet activating factor from radioactive acetate by stimulated platelets

H. Chap*, G. Mauco*, M. F. Simon*, J. Benveniste† & L. Douste-Blazy*

* INSERM Unité 101, Hôpital Purpan, 31059 Toulouse, France
† INSERM Unité 200, 32 rue des Carnets, 92140 Clamart Paris, France

Platelet activating factor (PAF-acether) is a chemical mediator released by basophils *in vitro* and *in vivo* conditions of immunological challenge^{1–5}. PAF-acether induces aggregation and secretion of platelets from different species^{3,6,7} and could thus be implicated in various immunological forms of tissue injury^{8,9}. Other sources have been described for this mediator, including macrophages¹⁰ and platelets¹¹. As the amounts of PAF-acether released in the latter case are sufficient to support the platelet aggregation¹¹ and as platelet aggregation by PAF-acether does not require ADP secretion or thromboxane biosynthesis¹², PAF-acether was proposed as the mediator of the putative third pathway of platelet aggregation¹¹. Structural analysis of PAF-acether by lipases indicated that it could be a glycerophospholipid devoid of an ester linkage at the 1-position and bearing a fatty acid esterified at the 2-position¹³. This has provided the first evidence for a phospholipid displaying the properties of a powerful chemical mediator. Based on several

When this reaction mixture was reduced, *trans*-4-hydroxyproline was generated. The experiments with hydroxyl radical scavengers, coupled with the fact that no hydroxylations occurred in the absence of added oxygen derivatives, indicate that some form of a reduced oxygen derivative is essential for the hydroxylation reaction to occur. The dependence of the reaction on the presence of metal ions is consistent with a reaction mechanism involving hydroxyl radicals, although the precise nature of the reactants in such mixtures is not understood¹⁹.

Reduced oxygen derivatives have been reported in widely differing circumstances in nature. Of particular interest to matrix biology is the finding that a major effect of these oxygen radicals is to alter extracellular macromolecules. For example, covalent cross-linking via dityrosines in sea urchin fertilization membranes²⁰, marine mussel byssal fibres²¹, collagen²², elastin²³ and resilin²⁴ have been reported. Depolymerization of hyaluronic acid has been observed by viscometry following exposure to oxygen radicals²⁵. Recently, it has been shown that plasma exposed to superoxide becomes chemotactic for neutrophils²⁶; our data suggest that the chemotactic response of fibroblasts and monocytes to hydroxyproline-containing peptides²⁷ may occur *in vivo* in the absence of collagen breakdown at, for example, sites of inflammation where reduced oxygen derivatives, which we have now shown can hydroxylate non-collagenous peptides, are being produced^{12,19,26}.

The present data indicate that hydroxyproline- and/or hydroxylysine-containing peptides could be produced *in vivo* by a nonenzymatic reaction involving reduced oxygen derivatives. If this is supported by further studies, then some of the isomers or hydroxyproline which are generated, such as the

lines of evidence, 1-*O*-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (1-alkyl-2-acetyl-GPC) was recently proposed as the chemical structure of the mediator, hence the term PAF-acether^{14,15}. As PAF-acether synthesis occurs rather early in activated platelets, one of the most efficient pathways of PAF-acether formation could involve a rapid acetylation of the corresponding lysoderivative (1-alkyl-GPC). We now present evidence that platelets activated by ionophore A23187 are able to incorporate acetate into a mixture of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC; the former supports the biological activity of PAF-acether.

Rabbit blood was drawn on to EDTA (5 mM) by intracardiac puncture and washed platelets were prepared according to Ardlie *et al.*¹⁶. Final suspension (10^9 platelets ml^{-1}) was in Tyrode's buffer (pH 7.35) containing 1 mM MgCl_2 , 1.25 mM CaCl_2 and 0.35% (w/v) fat-free bovine serum albumin (Sigma). Platelets were incubated at 37 °C for 10 min under stirring in the presence of 250 $\mu\text{Ci ml}^{-1}$ ^3H -acetate (12 Ci mmol^{-1} , CEA) and 2.5 μM A23187 (Lilly). Lipids were extracted according to Bligh and Dyer¹⁷. The lower chloroformic phase was washed three times with methanol/water (1:1 by vol.) to remove excess free acetate. An aliquot of the lipid extract from both control and A23187-treated platelets was subjected to TLC on silicagel F 254 precoated plates (Merck) using chloroform/methanol/water (70:35:7 by vol.) as a solvent. The chromatogram was divided into 17 bands which were directly scraped into scintillation vials containing 10 ml of Picofluor (Packard) and radioactivity was determined by scintillation counting. Figure 1 shows that A23187 induced the appearance of a radioactive lipid migrating very close to sphingomyelin. This chromatographic behaviour is identical to that previously reported for PAF-acether¹¹.

To characterize it further, the newly formed radioactive compound was then purified from a similar lipid extract by two steps of HPLC with a Lichrosorb Si 60/5 column (25 cm $\times$ 0.9 cm) at a flow rate of 5 ml min^{-1} . Using acetonitrile/methanol/water (60:30:10 by vol.) as a first solvent, a

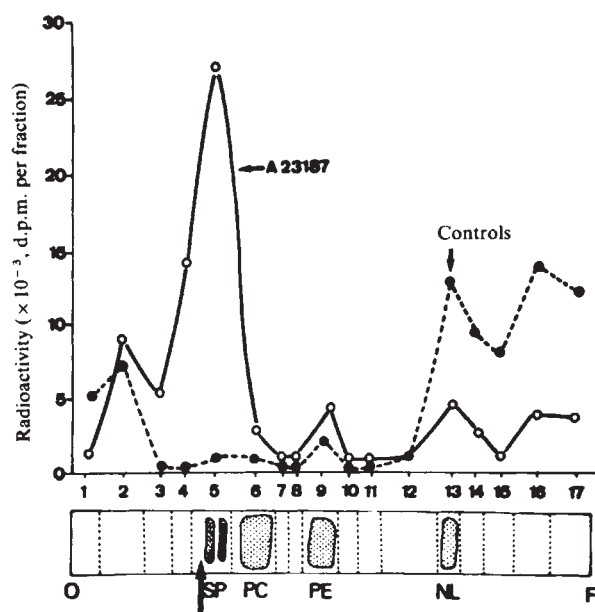


Fig. 1 Distribution of radioactivity after TLC of ^3H -acetate-labelled lipids from platelets. The lipid extracts from control (●) and A23187-treated (○) platelets were chromatographed as described in the text. The lower part of the graph represents the position of major, iodine-stainable lipids and the localization of the different bands scraped into scintillation vials. Each point represents the radioactivity measured in the corresponding band. Results are representative of four identical experiments. O, origin; SP, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; NL, neutral lipids; F, solvent front. The arrow indicates the position of a pure standard of PAF-acether.

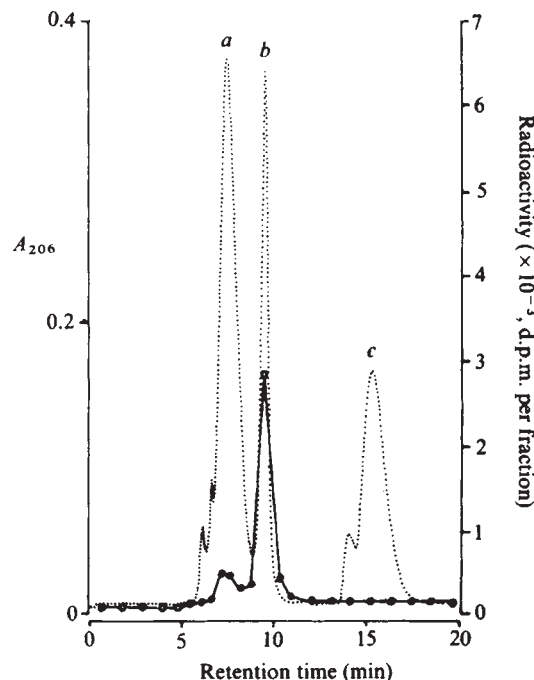


Fig. 2 Identification by HPLC of acetic acid as the hydrosoluble compound released by phospholipase A_2 treatment of purified ^3H -acetate-labelled platelet phospholipid. Preparation of ^3H -acetate-labelled platelet phospholipids has been reported in the text. An aliquot (40,000 d.p.m.) was incubated at 37 °C for 15 min with 5 IU *N. naja* phospholipase A_2 as previously reported¹³. After partition according to Bligh and Dyer¹⁷, to 80 μl of the aqueous upper layer were added 5 μl of pure formic acid (a), 5 μl acetic acid (b) and 10 μl propionic acid (c). The mixture was then subjected to HPLC using a column of RP 18-10 μm (25 $\times$ 0.46 cm). Elution was performed according to Farinotti *et al.*¹⁹ at a flow rate of 0.5 ml min^{-1} and 0.5-ml fractions were collected. Carrier acids were detected by absorbance at 206 nm (.....) and radioactivity (—) was determined by liquid scintillation counting.

radioactive peak was eluted at 23 min. The pooled fractions (which corresponded to PAF-acether on TLC) were rechromatographed with hexane/propanol 2/water (39:52:9 by vol.). In the latter system, a radioactive peak was detected at 30 min and was clearly separated from other UV-detectable lipids. On TLC, the purified compound exhibited the same R_F as previously observed and it co-migrated with 1-alkyl-2- ^3H -acetyl-GPC and 1-acyl-2- ^3H -acetyl-GPC (see Table 1 legend for their synthesis).

The biological activity of the purified compound was checked on rabbit platelets suspended in Tyrode's buffer (pH 7.35) containing gelatin (0.25%, w/v) as previously described³. The compound displayed a strong aggregating activity, which still occurred in the presence of creatine phosphate/creatine phosphokinase (4 mM and 10 U ml^{-1} , respectively) or when using platelets incubated for 15 min with 1 mM aspirin before washing (in these conditions, platelets became unresponsive to 10 μM sodium arachidonate).

These results strongly suggest that stimulated platelets synthesize radioactive PAF-acether from labelled acetate. Further biochemical identification was performed by subjecting the purified compound to various treatments. After base-catalysed methanolysis¹⁸ followed by neutralization with HCl and phase partition according to Bligh and Dyer¹⁷, over 90% of the radioactivity was found in the aqueous upper layer. The same result was obtained on incubation with *Naja naja* phospholipase A_2 in previously reported conditions¹³. In these conditions, it was verified by HPLC¹⁹ that the aqueous radioactivity coincided with free acetic acid (Fig. 2). These data confirm that acetic acid was esterified in the 2-position of the isolated phospholipid. However, incubation of the compound

Table 1 Percentage of hydrosoluble radioactivity after lipase treatment of ^3H -acetate-labelled phospholipids

	No. of experiments	Controls	Lipase	P
Platelet ^3H -phospholipid	6	16.2 $\pm$ 7.1	60.0 $\pm$ 9.6	<0.001
1-acyl-2-[^3H]acetyl-GPC	5	16.2 $\pm$ 8.0	79.5 $\pm$ 4.9	<0.001
1-alkyl-2-[^3H]acetyl-GPC	6	29.4 $\pm$ 9.7	30.9 $\pm$ 8.6	NS

The ^3H -acetate-labelled phospholipid isopolar to PAF-acether was purified from a platelet lipid extract as described in the text. 1-Acyl-GPC was obtained by phospholipase A_2 treatment (*Crotalus adamanteus*) of phosphatidylcholine isolated from egg²⁶ and then purified by TLC. This and synthetic 1-hexadecyl-*rac*-glyceryl-3-phosphorylcholine (Medmark) were acetylated as described by Demopoulos *et al.*¹⁴ in chloroform containing ^3H -acetic anhydride (Amersham) diluted with cold anhydride to a specific radioactivity of 1 mCi μmol^{-1} . The two products were then purified by HPLC. Lipase treatment was performed at 37 °C for 60 min in 0.01 M Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.01 M CaCl_2 . The incubation medium contained in 0.25 ml: 0.02 ml of labelled phospholipid in ethanol (20,000–40,000 d.p.m.) and 0.025 ml of either lipase from *R. arrhizus* (Boehringer) suspended in 3.2 M $(\text{NH}_4)_2\text{SO}_4$ or 3.2 M $(\text{NH}_4)_2\text{SO}_4$ in the control tubes. Lipids were then partitioned according to Bligh and Dyer¹⁷ and an aliquot of both lower (organic) and upper (aqueous) phase was determined for radioactivity by liquid scintillation counting using Picofluor (Packard). Results (mean $\pm$ 1 s.d.) represent the percentage of radioactivity recovered in the upper phase. P, probability of significance according to Student's *t*-test. NS, not significant.

with 4 M HCl at room temperature for 3 h (ref. 5) did not modify its phase partition, excluding the occurrence of a vinyl-ether linkage at the 1-position of the phospholipid.

More striking results were obtained following treatment of the radiolabelled compound by lipases. As shown in Table 1, 60% of the radioactivity was water soluble after incubation with lipase from *Rhizopus arrhizus*. This value was close to 80% when incubating 1-acyl-2-[^3H]acetyl-GPC, whereas no change in phase partition occurred with 1-hexadecyl-2-[^3H]acetyl-GPC. Identical behaviour was obtained when using a lipase with high phospholipase A_1 activity purified from guinea pig pancreas (donated by J. Fauvel and M. J. Bonnefis²⁰). These results indicate that the radioactive phospholipid that we isolated from A23187-stimulated platelets could correspond to a mixture of 1-acyl-2-acetyl-GPC and 1-alkyl-2-acetyl-GPC, the former being converted to hydrosoluble 2-acetyl-GPC through lipase hydrolysis. Indeed, TLC results have shown that the water-soluble compound released by lipase treatment of the ^3H -labelled platelets was the same as that obtained from 1-acyl-2-[^3H]acetyl-GPC. It remained at the origin and was clearly separated from PAF-acether and from free acetate, the latter migrating with the solvent front. In contrast the chromatographic behaviour of 1-alkyl-2-acetyl-GPC was unchanged after lipase treatment, thereby illustrating the specificity of this enzyme towards the ester bond at the 1-position of phosphoglycerides²¹. However, the lower organic phase contained all the original biological activity after such treatment, confirming previous data obtained on PAF-acether from pig leukocytes¹³. We therefore conclude that only 1-alkyl-2-acetyl-GPC supports the PAF-acether activity released by A23187-stimulated platelets. This agrees with the finding that the 1-acyl derivative exhibits a much lower activity than the 1-alkyl derivative^{14,15}.

The present study is an attempt to characterize the biochemical pathways of PAF-acether synthesis in a living cell. More recent results indicate that a similar incorporation of radiolabelled acetate occurs in stimulated murine macrophages²². However, several aspects of acetate incorporation in platelet PAF-acether remain unclear: (1) Are the concentrations of acetate used (20 μM) relevant to available intracellular acetate? Our study does not provide quantitative information; we simply expected the radioactive precursor to label the intracellular pool of acetate, the precise amount of which is

unknown. (2) Is the acetylation reaction an enzymatic process involving, for instance, acetylcoenzyme A as an intermediate? This should be the object of further studies. (3) What is the origin of the phospholipid acceptors? 1-alkyl-GPC is released from hog leukocytes²³ and various other cell sources²⁴, including platelets, but we know of no accurate published report on the putative precursor 1-alkyl-2-acyl-GPC in these cells. The 1-alkyl- and 1-acyl-lysophospholipids could then act as acetyl acceptors for acetate, leading to the formation of a molecule with high or low activity. This would agree with the suppression of PAF-acether synthesis by bromophenacyl bromide, a phospholipase A_2 inhibitor²⁵. However, further studies are necessary to characterize the mechanism and the specificity of acetate incorporation into these phospholipids.

This work was supported by INSERM (CRL 78.5.148.3). We thank Dr L. McGregor for correcting the manuscript.

Received 23 July; accepted 31 October 1980.

1. Siraganian, R. P. & Osler, A. G. *J. Immun.* **106**, 1244–1251 (1971).
2. Henson, P. M. & Cochrane, C. J. *J. exp. Med.* **133**, 554–571 (1971).
3. Benveniste, J., Henson, P. M. & Cochrane, C. G. *J. exp. Med.* **136**, 1356–1377 (1972).
4. Benveniste, J. *Nature* **249**, 581–583 (1974).
5. Pinckard, R. N., Farr, R. S. & Hanahan, D. J. *J. Immun.* **123**, 1847–1857 (1979).
6. Benveniste, J., Le Couedic, J. P. & Kamoun, P. *Lancet* **i**, 344 (1975).
7. Fesus, L., Csaba, B. & Muszbek, L. *Clin. exp. Immun.* **27**, 512–515 (1977).
8. Henson, P. M. & Pinckard, R. N. *Monogr. Allergy* **12**, 13–26 (1977).
9. Benveniste, J., Camussi, J. & Polonsky, J. *Monogr. Allergy* **12**, 138–142 (1977).
10. Mencia-Huerta, J. M. & Benveniste, J. *Eur. J. Immun.* **9**, 409–415 (1979).
11. Chignard, M., Le Couedic, J. P., Tence, M., Vargaftig, B. B. & Benveniste, J. *Nature* **279**, 799–800 (1979).
12. Cazenave, J. P., Benveniste, J. & Mustard, J. F. *Lab. Invest.* **41**, 275–285 (1979).
13. Benveniste, J., Le Couedic, J. P., Polonsky, J. & Tence, M. *Nature* **269**, 170–171 (1977).
14. Demopoulos, C. A., Pinckard, R. N. & Hanahan, D. J. *J. biol. Chem.* **254**, 9355–9358 (1979).
15. Benveniste, J. *et al. Cr. heb. Acad. Sci., Paris* **289**, 1037–1040 (1979).
16. Ardlie, N. G., Packham, M. A. & Mustard, J. F. *Br. J. Haemat.* **19**, 7–17 (1970).
17. Bligh, E. G. & Dyer, W. J. *Can. J. Biochem. Physiol.* **37**, 911–918 (1959).
18. Tence, M., Polonsky, J., Le Couedic, J. P. & Benveniste, J. *Biochimie* **62**, 252–259 (1980).
19. Farinotti, R., Cade, M., Mahuzier, G. & Rosset, R. *Analisis* **7**, 449–453 (1979).
20. Fauvel, J. & Bonnefis, M. J. (in preparation).
21. Slotboom, A. J., de Haas, G. H., Bensen, P. P. M., Burbach-Westerhuis, G. J. & van Deenen, L. L. M. *Chem. Phys. Lipids* **4**, 15–29 (1970).
22. Mencia-Huerta, J. M., Roubin, R. & Benveniste, J. (in preparation).
23. Polonsky, J., Tence, M., Varenne, P., Das, B., Lunel, J. & Benveniste, J. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
24. Benveniste, J. (in preparation).
25. Mencia-Huerta, J. M., Akerman, C. & Benveniste, J. *Fedn. Proc.* **39**, 691 (1980).
26. Papahadjopoulos, D. & Miller, M. *Biochim. biophys. Acta* **135**, 624–638 (1967).

Effects of ATP and vanadate on calcium efflux from barnacle muscle fibres

Mark T. Nelson & Mordecai P. Blaustein

Department of Physiology, University of Maryland, School of Medicine, Baltimore, Maryland 21201
Department of Physiology and Biophysics, Washington University, School of Medicine, St Louis, Missouri 63110

Calcium ions carry the inward current during depolarization of barnacle muscle fibres and are involved in the contraction process¹. Intracellular ionized calcium ($[\text{Ca}^{2+}]_i$) in barnacle muscle, as in other cells, is kept at a very low concentration, against a large electrochemical gradient^{2,3}. This large gradient is maintained by Ca^{2+} extrusion mechanisms. When $[\text{Ca}^{2+}]_i$ is below the contraction threshold⁴, Ca^{2+} efflux from giant barnacle muscle fibres is, largely, both ATP dependent and external Na^+ (Na_o^+) dependent (see also refs 5,6). When $[\text{Ca}^{2+}]_i$ is raised to the level expected during muscle contraction (2–5 μM)⁷, most of the Ca^{2+} efflux from perfused fibres is Na_o^+ dependent; as in squid axons⁸, this Na_o^+ -dependent Ca^{2+} efflux is ATP independent. Orthovanadate is an inhibitor of $(\text{Na}^+ + \text{K}^+)$ ATPase⁹ and the red cell Ca^{2+} -ATPase¹⁰. We report here that vanadate inhibits ATP-promoted, Na_o^+ -dependent Ca^{2+} efflux from barnacle muscle fibres perfused with low $[\text{Ca}^{2+}]_i$ (0.2–0.5 μM), but has little effect on the Na_o^+ -dependent, ATP-independent Ca^{2+} efflux from fibres with a high $[\text{Ca}^{2+}]_i$ (2–

5 μM). Nevertheless, ATP depletion or vanadate treatment of high $[\text{Ca}^{2+}]_i$ fibres causes an approximately 50-fold increase of Ca^{2+} efflux into Ca^{2+} -containing lithium seawater. These results demonstrate that both vanadate and ATP affect Ca^{2+} extrusion, including the Na_o^+ -dependent Ca^{2+} efflux (Na-Ca exchange), in barnacle muscle.

The intracellular solute composition of giant muscle fibres from the barnacle, *Balanus nubilus*, was controlled by internal perfusion¹¹. Intracellular $[\text{Ca}^{2+}]_i$ was maintained with Ca-EGTA buffers containing 2 or 8 mM EGTA and appropriate concentrations of CaCl_2 (see figure legends). The perfusion fluids also contained caffeine, and the mitochondrial uncoupler, carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), to minimize Ca^{2+} sequestration in sarcoplasmic reticulum and mitochondria, respectively. Caffeine and FCCP do not directly affect Ca^{2+} efflux from internally perfused barnacle muscle fibres (unpublished data). ATP levels are controlled with a regenerating system consisting of phosphoenolpyruvate and phosphokinase. Perfused fibres do not contract even when $[\text{Ca}^{2+}]_i$ is raised above the normal contraction threshold ($\sim 0.9 \mu\text{M}$)⁴. Thus, Ca^{2+} efflux measurements can be made on fibres containing Ca^{2+} levels that would normally be expected to cause contractures ($> 1 \mu\text{M}$)⁷.

As in squid axons⁸, Na_o^+ -dependent Ca^{2+} efflux from barnacle muscle fibres is promoted by ATP when $[\text{Ca}^{2+}]_i < 1 \mu\text{M}$ (ref. 5). Ca efflux from ATP-depleted fibres perfused with 0.2–0.5 μM $[\text{Ca}^{2+}]_i$ was $\sim 0.3 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ($n = 3$) and insensitive to the removal of external Na^+ . Similarly, Ca^{2+} efflux from ATP-fuelled, vanadate-treated fibres perfused with 0.2–0.5 μM $[\text{Ca}^{2+}]_i$ was $\sim 0.2 \text{ pmol cm}^{-2} \text{ s}^{-1}$ ($n = 3$) and was insensitive to the removal of external Na^+ . However, as shown in Fig. 1, in a representative fibre perfused with ATP and 0.2 μM $[\text{Ca}^{2+}]_i$, Ca^{2+} efflux was about $0.75 \text{ pmol cm}^{-2} \text{ s}^{-1}$. This efflux declined to about $0.35 \text{ pmol cm}^{-2} \text{ s}^{-1}$ when external Na^+ was replaced by

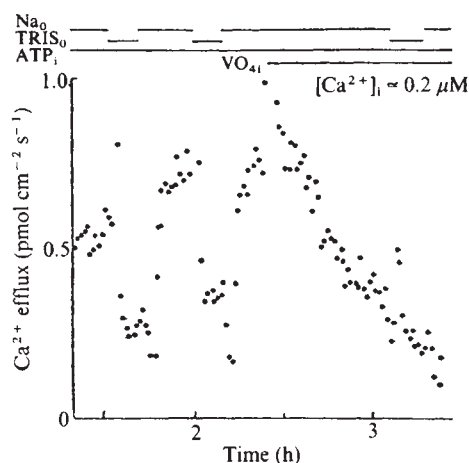


Fig. 1 Effects of external Na^+ and Tris, and internal vanadate on $^{45}\text{Ca}^{2+}$ efflux from a fibre perfused with low $[\text{Ca}^{2+}]_i$. The composition of the perfusion fluid was: 189 mM K glutamate, 10 mM Na glutamate, 38 mM KCl, 375 mM sucrose, 8 mM MgCl_2 , 8 mM EGTA, 4.1 mM CaCl_2 ($[\text{Ca}^{2+}]_i \approx 0.2 \mu\text{M}$), 3 mM Na_2ATP (Sigma), 3 mM phosphoenolpyruvate (Sigma), 8 $\mu\text{g ml}^{-1}$ pyruvate kinase, and 40 mM HEPES. The perfusion fluids were buffered to pH 7.3 with Tris base. A Ca-EGTA stability constant of $7.6 \times 10^6 \text{ M}^{-1}$ was used to calculate free calcium concentrations ($[\text{Ca}^{2+}]_i$). The composition of the standard external solution (Na_o^+) was (in mmol l^{-1}): 456 NaCl, 6 Tris, 10 KCl, 11 CaCl_2 and 32 MgSO_4 . All external solutions were buffered to pH 7.8 at 20°C with maleic acid. Osmolalities of all internal and external solutions were 930–980 mosmol kg^{-1} . Tris or Li^+ (see Figs 2 and 3) was substituted isosmotically for Na^+ in the Na-free seawaters (indicated by Tris or Li_o in the bars at the tops of the figures). Internal perfusion with ^{45}Ca , 2 mM caffeine, and 25 μM FCCP was begun 80 min before the data shown here were obtained. After 150 min of perfusion, 1 mM vanadate was added to the perfusion fluid. Resting potential, -56 mV . Temperature, 16°C . Fibre diameter, 1.4 mm.

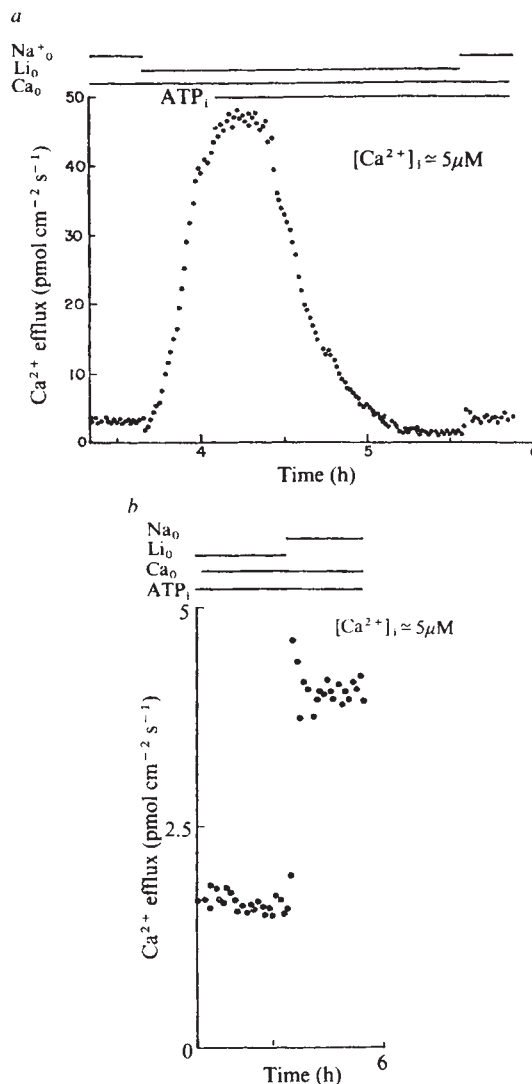


Fig. 2 *a*, Effects of external Na^+ and Li^+ , and internal ATP on $^{45}\text{Ca}^{2+}$ efflux from a barnacle muscle fibre perfused with high $[\text{Ca}^{2+}]_i$. *b*, Effects of external Na^+ and Li^+ on $^{45}\text{Ca}^{2+}$ efflux from the same fibre in the presence of ATP (last part of Fig. 2*a* expanded). Internal perfusion with fluid containing $^{45}\text{Ca}^{2+}$, 2 mM caffeine, 25 μM FCCP, 2 mM EGTA and 1.95 mM Ca ($[\text{Ca}^{2+}]_i \approx 5 \mu\text{M}$) was begun 200 min before data shown here were obtained. After 250 min of perfusion, 2 mM ATP and an ATP-regenerating system were added to the perfusion fluid. The fibre was exposed to 456 mM Na^+ seawater, except during the periods indicated, when the seawater contained 456 mM Li^+ . Resting potential, -47 mV . Temperature, 16°C . Fibre diameter, 1.4 mm.

Tris. When 1 mM vanadate was added to the perfusion fluid, Ca^{2+} efflux into Na^+ seawater fell to $\sim 0.2 \text{ pmol cm}^{-2} \text{ s}^{-1}$. These data demonstrate that Ca^{2+} efflux from fibres containing low $[\text{Ca}^{2+}]_i$ is Na_o^+ dependent, and can be inhibited by vanadate.

In fibres containing a high $[\text{Ca}^{2+}]_i$, bathed in Ca^{2+} -free media, Li^+ and Tris (and choline) could be used interchangeably as external Na^+ replacements (compare with Fig. 2). However, in fibres with a low $[\text{Ca}^{2+}]_i$, Li^+ seems to substitute partially for external Na^+ in supporting Ca^{2+} efflux¹². Therefore, Tris was used to replace Na^+ in fibres with a low $[\text{Ca}^{2+}]_i$ (Fig. 1).

In squid axons containing high $[\text{Ca}^{2+}]_i$ ($> 2 \mu\text{M}$), most of the Ca^{2+} efflux is Na_o^+ dependent, but ATP independent⁸. In addition, in high $[\text{Ca}^{2+}]_i$, ATP-depleted axons⁸, the replacement of external Na^+ by Li^+ increases Ca^{2+} efflux two- to fourfold. This Li_o^+ -stimulated efflux is also completely dependent on external Ca^{2+} . Ca^{2+} efflux from perfused barnacle muscle fibres exhibits similar properties. Namely, when ATP-depleted fibres are perfused with high $[\text{Ca}^{2+}]_i$, the replacement of external Na^+ by Li^+ increases Ca^{2+} efflux about 10-fold (from ~ 5 to $\sim 50 \text{ pmol}$

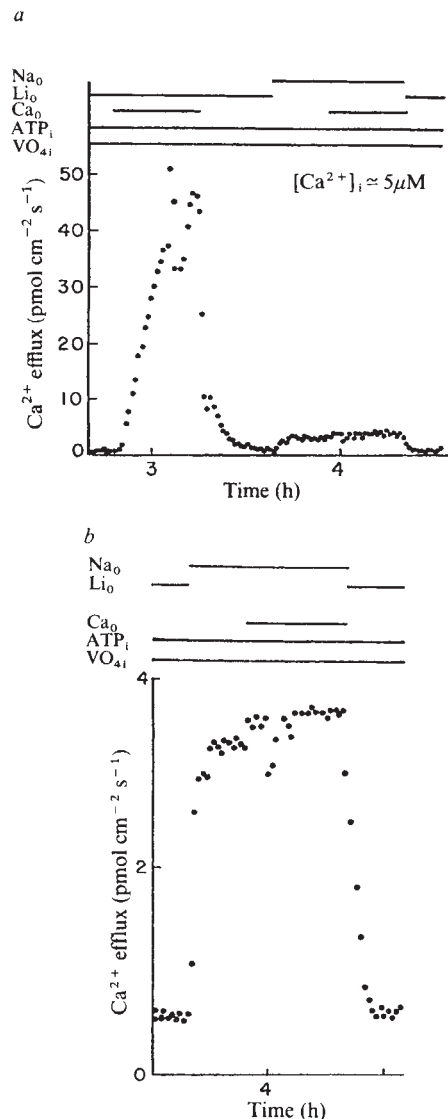


Fig. 3 *a* and *b*, Effects of external Na^+ , Li^+ , and Ca^{2+} on $^{45}\text{Ca}^{2+}$ efflux from an ATP-fuelled fibre perfused with high $[\text{Ca}^{2+}]_i$. The end tail of the experiment of *a* is shown on an expanded scale in *b*. Internal perfusion with $^{45}\text{Ca}^{2+}$, 3 mM ATP and an ATP-regenerating system, 1.2 mM vanadate, 2 mM caffeine, 8 mM EGTA and 7.8 mM Ca^{2+} ($[\text{Ca}^{2+}]_i \approx 5 \mu\text{M}$) was begun 160 and 210 min before data shown in *a* and *b*, respectively, were obtained. The fibre was bathed in 456 mM Na^+ , except for the periods noted, when the seawater contained 456 mM Li^+ . The seawater also contained 11 mM Ca^{2+} , except for the periods indicated, when Mg^{2+} replaced Ca^{2+} in the seawater. Resting potential, -45 mV . Temperature, 16°C . Fibre diameter, 1.5 mm.

$\text{cm}^{-2} \text{s}^{-1}$; see Fig. 2*a*). This Li_0^+ -stimulated efflux is also entirely Ca_0^{2+} dependent—the removal of Ca_0^{2+} reduces Ca^{2+} efflux into Li^+ seawater (Li SW) to $\sim 1 \text{ pmol cm}^{-2} \text{s}^{-1}$ (not shown). Moreover, the addition of ATP to the perfusate reduces Li_0^+ + Ca_0^{2+} -dependent Ca^{2+} efflux (Fig. 2*a*), and the subsequent replacement of Li_0^+ by Na_0^+ increases Ca^{2+} efflux to about the level seen in the absence of ATP ($\sim 4 \text{ pmol cm}^{-2} \text{s}^{-1}$; Fig. 2*a, b*).

Vanadate mimics the effect of ATP depletion in ATP-fuelled fibres perfused with high $[\text{Ca}^{2+}]_i$: it promotes a large Ca^{2+} efflux in Li SW (Fig. 3*a*; compare with Fig. 2*a*). Li_0^{2+} -stimulated Ca^{2+} efflux from vanadate-treated, ATP-fuelled fibres, like Li_0^{2+} -stimulated Ca^{2+} efflux from ATP-depleted fibres, is completely dependent on Ca_0^{2+} (Fig. 3*a*).

Figure 3*a, b* also shows that Ca^{2+} efflux from vanadate-treated, high $[\text{Ca}^{2+}]_i$ fibres can be promoted by Na_0^+ in the absence of Ca_0^{2+} . In summary, the data from Figs 1 and 3*a*

demonstrate that the Na_0^+ -dependent Ca^{2+} efflux (presumably Na^+ - Ca^{2+} exchange) is blocked by vanadate when it is ATP dependent (in low $[\text{Ca}^{2+}]_i$ fibres), but not when it is ATP independent (in high $[\text{Ca}^{2+}]_i$ fibres)¹¹. Clearly, vanadate cannot be used to distinguish Na_0^+ -dependent from Na_0^+ -independent Ca^{2+} efflux (contrast with ref. 13).

Resting barnacle muscle fibres have a $[\text{Ca}^{2+}]_i$ of about 0.05–0.2 μM (ref. 14). The threshold for contraction is about 0.5–0.9 μM (ref. 4), and maximal isometric force is developed with a 10-fold higher $[\text{Ca}^{2+}]_i$ (ref. 7). Our data demonstrate that the Ca^{2+} efflux is Na_0^+ -dependent throughout the normal physiological range of $[\text{Ca}^{2+}]_i$ values for these fibres. When $[\text{Ca}^{2+}]_i$ is below the contraction threshold ($< \sim 0.5 \mu\text{M}$)⁴, the Ca^{2+} efflux is largely ATP dependent and vanadate sensitive. A substantial fraction of this efflux ($\sim 60\%$) depends on Na_0^+ when $[\text{Ca}^{2+}]_i \approx 0.2 \mu\text{M}$ (Fig. 1). At the $[\text{Ca}^{2+}]_i$ levels expected at the peak of contraction ($\sim 5 \mu\text{M}$)⁷, about 90% of the Ca^{2+} efflux is Na_0^+ dependent; but this (Na_0^+ -dependent) efflux is neither ATP dependent nor vanadate sensitive.

The mechanism responsible for the residual Ca^{2+} efflux into Na^+ -free media is uncertain. This efflux could, in part, be a passive Ca^{2+} and Ca -EGTA leak. However, Ca^{2+} efflux from ATP-fuelled fibres into Na^+ -free media was greater than Ca^{2+} efflux from ATP-depleted or vanadate-treated fibres (even with external Na^+ present; compare with Fig. 1). This difference in efflux may represent an ATP-dependent, vanadate-sensitive 'uncoupled' Ca^{2+} pump¹³. Alternatively, this efflux may simply represent an exchange that is activated by other monovalent cations (for example, K^+ , Li^+ , choline or Tris)¹² and/or by the residual external Na^+ located in the spaces enclosed by surface membrane invaginations.

Our experiments do not show how ATP affects Na^+ - Ca^{2+} exchange. However, as vanadate inhibits a number of ATPases¹⁵, our data may infer that the influence of ATP on Na_0^+ -dependent Ca^{2+} efflux involves a phosphorylation step. This is consistent with evidence that the non-hydrolysable β - γ methylene analogue of ATP cannot substitute for ATP in promoting Ca^{2+} efflux from the squid axons¹⁶. Unfortunately, ATP hydrolysis possibly associated with Na^+ - Ca^{2+} exchange would be difficult to detect in barnacle muscle fibres which contain many ATPases.

Our data are consistent with the view⁸ that ATP increases the affinity of the Na^+ - Ca^{2+} exchange system for external Na^+ and/or internal Ca^{2+} . Moreover, the effects of ATP and vanadate on the Ca^{2+} efflux in high $[\text{Ca}^{2+}]_i$ fibres may indicate that ATP increases the Na^+ / Li^+ selectivity ratio of the carriers, thereby inhibiting Li_0^+ + Ca_0^{2+} -dependent Ca^{2+} efflux. Regardless of the uncertainties, our data demonstrate that Na^+ ions, probably acting via an Na^+ - Ca^{2+} exchange mechanism, are important for regulating Ca^{2+} in barnacle muscle fibres in physiological conditions.

We thank Dr E. Santiago for technical assistance, Dr L. Reuss for a supply of orthovanadate, and Mrs B. Brooks for preparing the typescript. This research was supported by MDAA, NINCDS, and NSF. M.T.N. was supported by an NIH training grant.

Received 25 August; accepted 30 September 1980.

1. Atwater, I. *et al.* *J. Physiol. Lond.* **243**, 523–551 (1974).
2. Baker, P. F. *Ann. N.Y. Acad. Sci.* **307**, 250–268 (1978).
3. Blaustein, M. P. *Rev. physiol. biochem. Pharmac.* **70**, 34–82 (1974).
4. Ashley, C. C. *Am. Zool.* **7**, 647–659 (1967).
5. DiPolo, R. & Caputo, C. *Biochim. biophys. Acta* **470**, 389–394 (1977).
6. Russell, J. M. & Blaustein, M. P. *J. gen. Physiol.* **63**, 144–167 (1974).
7. Ashley, C. C. *Ann. N.Y. Acad. Sci.* **307**, 308–329 (1978).
8. Blaustein, M. P. *Biophys. J.* **20**, 79–111 (1977).
9. Cantley, L. C. *et al.* *J. biol. Chem.* **252**, 7421–7422 (1977).
10. Bond, G. H. & Hudgins, P. M. *Fedn Proc.* **37**, 315 Abstr. (1978).
11. Nelson, M. T. & Blaustein, M. P. *J. gen. Physiol.* **75**, 183–206 (1980).
12. DiPolo, R. *et al.* *J. gen. Physiol.* **67**, 433–467 (1976).
13. DiPolo, R. *et al.* *Nature* **281**, 228–229 (1979).
14. Hagiwara, S. & Nakajima, S. *J. gen. Physiol.* **49**, 807–818 (1966).
15. Simons, T. J. B. *Nature* **281**, 337–338 (1979).
16. DiPolo, R. *J. gen. Physiol.* **69**, 795–813 (1977).

Co-release of enkephalin and catecholamines from cultured adrenal chromaffin cells

Bruce G. Livett, Deanne M. Dean, Larry G. Whelan, Sidney Udenfriend* & Jean Rossier*†

Division of Neurology, The Montreal General Hospital and McGill University, Montreal, Quebec, Canada H3G 1A4

* Roche Institute of Molecular Biology, Nutley, New Jersey 07110

The opioid peptides Leu-enkephalin and Met-enkephalin^{1,2} are stored intraneuronally in the brain³ where they are thought to act as neurotransmitters and/or neuromodulators⁴. Evidence for their release from nerve terminals has come from biochemical and pharmacological studies *in vitro* with brain tissue slices^{5,6} and synaptosomes⁶. Enkephalins also exist in the peripheral nervous system in nerve cell bodies and axon terminals in the gastrointestinal tract⁷, sympathetic ganglia⁸⁻¹¹ and adrenal gland¹². In the adrenal gland, high levels of enkephalins are present both in axon terminals of the splanchnic nerve¹³ and in the adrenal medullary chromaffin cells¹³⁻¹⁵ where they are stored together with the catecholamines in the chromaffin granules¹⁴⁻¹⁶. Stimulation of the adrenal gland *in vivo*^{15,17} or the perfused gland *in vitro*¹⁴ causes release of catecholamines and enkephalins into the adrenal vein. However, it is not clear whether the origin of the released enkephalins is the adrenal medullary chromaffin cells or the enkephalin-containing splanchnic nerve terminals that innervate the medulla. We now show that enkephalin and catecholamines are released together from primary cultures of bovine adrenal medullary chromaffin cells by nicotine in a Ca²⁺-dependent manner.

Primary cultures of bovine adrenal medullary chromaffin cells provide a useful experimental system for investigating the release of catecholamines uncomplicated by diffusion barriers present in intact tissue. For example, this cell culture system has been used to characterize the pharmacological release of catecholamines from the cells by nicotinic agonists¹⁸, to investigate the role of substance P^{18,19}, somatostatin¹⁹ and enkephalins²⁰ as modulators of these nicotinic receptors, and to demonstrate the presence of Leu- and Met-enkephalin in high concentrations in the processes and terminal varicosities that characterize these adrenal paraneurons²¹. For the present study, primary cell cultures of adrenal medullary cells were established as monolayers on collagen-coated plastic tissue culture dishes¹⁹ (for further details see Fig. 1 legend). After 8 days in culture, the cells were processed for immunohistochemical localization of Leu-enkephalin as previously described²¹. Preimmune rabbit serum, rabbit anti-Leu-enkephalin serum (R164B) preabsorbed with Met-enkephalin and fluorescein-conjugated goat anti-rabbit serum used in this study were obtained from Dr R. P. Elde. The specificity of the Leu-enkephalin antiserum has been characterized by radioimmunoassay and immunofluorescence²¹. Strong immunofluorescence specific for leu-enkephalin (Fig. 1b) was observed in the perikarya and varicose processes of approximately 30% of the adrenal paraneurons whereas over 80% of the cells stained positively for catecholamines (Fig. 1c) by the glyoxylic or formaldehyde-glutaraldehyde techniques^{18,21}. The immunoreactive Leu-enkephalin had a fine granular disposition within the cytoplasm and processes and exhibited an increasing proximal to distal ratio of fluorescence consistent with synthesis in the perikarya with subsequent transport, processing and storage in the terminals²¹.

Catecholamines are normally released from the adrenal gland *in vivo* following activation of acetylcholine (ACh) receptors on the chromaffin cells by ACh released from splanchnic nerve terminals innervating the medulla. In a similar manner, phar-

Table 1 Calcium-dependent release of Leu-enkephalin and catecholamines from adrenal paraneurons

Secretory stimulus	% Cellular content released		
	Noradrenaline	Adrenaline	Leu-enkephalin
Nicotine 5×10^{-6} M	1.4 ± 0.7	1.0 ± 0.2	ND
Nicotine 5×10^{-6} M + Ca ²⁺ , 2.2 mM	20.9 ± 1.4	10.4 ± 0.8	8.2 ± 1.3

The release studies were carried out on 10 culture plates (6 days old) each containing 1×10^6 chromaffin cells grown in the presence of Bacitracin (see Fig. 1 legend) in 1 ml medium. Five plates served as controls (nicotine but no Ca²⁺) and the other five contained nicotine and 2.2 mM Ca²⁺. Each plate was washed at 37 °C for 15 min in 1.0 ml of a HEPES-buffered Krebs-Ringer saline (KRH) containing 0.2% bovine serum albumin¹⁹ and the plates were then incubated in the release experiments for three successive 5-min intervals in: 1 ml of KRH medium alone (0–5 min); KRH medium containing nicotine (5–10 min); KRH medium alone, 'washout' sample (10–15 min). At the end of each 5-min period, the incubation media were removed from the cells, placed on ice and the new incubation medium pre-equilibrated to 37 °C added to the cells. A 100-μl aliquot of the medium removed from the cells was acidified with 25 μl 2 M perchloric acid for catecholamine estimation and the remaining 900 μl acidified with 900 μl 2 M acetic acid for subsequent extraction, chromatography and analysis of Leu-enkephalin. After 15 min the cells were scraped off the plate in 0.5 ml of ice-cold KRH with a rubber policeman and the extract acidified as above for assay of catecholamines and Leu-enkephalin. Assay of endogenous catecholamines: Endogenous catecholamines (both within the cells and released into the media) were assayed by the two-wavelength differential spectrofluorimetric procedure²⁵ of Renzini *et al.*²⁶. Noradrenaline and adrenaline levels were obtained by reference to standards after solving simultaneous equations for fluorescence of the samples at 410 and 455 nm with a program (written by Mr Serge La Fontaine) for a digital PDP 11/40 computer. The variability of triplicate assays of the same sample was $\pm 5\%$. These day-6 cultures contained 7.6 ± 0.8 ($n = 10$) μg adrenaline per 10^6 cells and 2.9 ± 0.5 ($n = 10$) μg noradrenaline per 10^6 cells (molar ratio adrenaline:noradrenaline = 2.4). Assay of endogenous Leu-enkephalin: The freeze-dried extracts of cells and media were reconstituted in 10 mM sodium phosphate buffer pH 7.5, 145 mM NaCl supplemented with thiomersal 0.01%, gelatine 0.1% and crystalline bovine serum albumen 0.01%. The extracts were assayed by a radioimmunoassay using a C-terminal-directed Leu-enkephalin antiserum. The characteristics of the C-terminal Leu⁵-enkephalin antiserum (RB 92) have been described elsewhere²³. This antiserum has a cross-reactivity of 3% with Met⁵-enkephalin, and is quite specific for the C-terminus of Leu-enkephalin. In terms of molar ratio it has a cross-reactivity of 3% with Met-enkephalin²³ and $<1/10,000$ with Leu-enkephalin-Arg⁶ and Met-enkephalin Arg⁶. Note that although the radioimmunoassay is highly selective for Leu-enkephalin, Met-enkephalin is generally present in amounts several times greater than Leu-enkephalin, and is also released¹⁵ and will therefore necessarily be detected in the radioimmunoassay. However, the Leu⁵-enkephalin immunoreactivity in the cell extracts and in the media was further characterized by gel filtration and HPLC and was found to correspond to authentic Leu⁵-enkephalin. The amount of endogenous catecholamines and Leu-enkephalin contained in the cells at the beginning of each experiment was estimated for each culture plate by summing the amounts in the media at the three time periods and adding it to that remaining in the cells at 15 min. The release of endogenous catecholamines and Leu-enkephalin from the cells into the medium during the 5-min stimulation period is expressed as a percentage of the total content in the cells at the beginning of the experiment. The results give the mean $\pm$ s.e.m. ($n = 5$) where the percentage of catecholamines and Leu-enkephalin released by nicotine stimulation has been compared by Students *t*-test. ND, not detected.

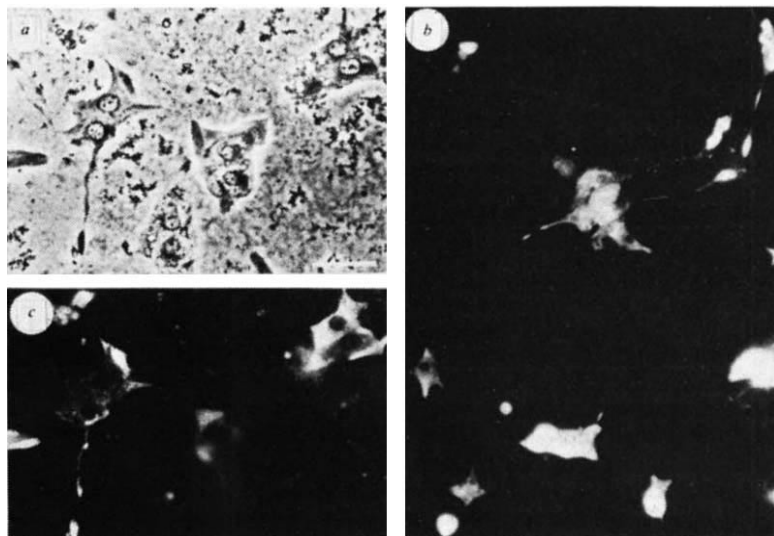
* $P < 0.001$; † not significant at $P < 0.2$.

macological studies on bovine adrenal paraneurons have shown that ³H-noradrenaline taken up and stored in reserpine-sensitive sites can be released from the cells by activation of nicotinic (but not muscarinic) ACh receptors¹⁹. The results in Table 1 show that in the presence of nicotine (ED₅₀ 5×10^{-6} M) the cells released $13.2 \pm 1.6\%$ ($n = 5$) of their endogenous total catecholamines over a 5-min period. Nicotine produced a preferential release of endogenous noradrenaline ($20.9 \pm 1.4\%$) over adrenaline ($10.4 \pm 0.8\%$). It also released enkephalins from the cells, and the fraction of the cellular Leu-enkephalin released ($8.2 \pm 1.3\%$) was similar to that of the endogenous adrenaline released. The release of both catecholamines and Leu-enkephalin by nicotine was Ca²⁺ dependent (Table 1). Although Met-enkephalin and enkephalin congeners and precursors contained in the chromaffin granules¹⁴⁻¹⁶ are also released^{15,22}, the combination of immunoassay gel filtration and HPLC methods^{22,23} used in our study were highly selective for Leu-enkephalin (see Table 1 legend).

A characteristic feature of the nicotinic receptor response is that it shows activation at low levels of agonist (10^{-7} – 10^{-5} M nicotine) and inhibition at higher levels ($>10^{-4}$ M). In accord

† Present address: Laboratoire de physiologie nerveuse, CNRS, 91190 Gif sur Yvette, France.

Fig. 1 Histochemical localization of Leu-enkephalin and catecholamines in the perikarya and varicose processes of bovine adrenal paraneurons maintained in cell culture for 6 days. *a*, Phase: background material is serum protein and collagen substrate precipitated by Bouin's fixative. *b*, Same field as *a*; Leu-enkephalin immunofluorescence. *c*, Catecholamine fluorescence. Bars represent 50 μm in *a*, *b*, 100 μm in *c*. Chromaffin cells were isolated from bovine adrenal medullae by retrograde perfusion with Collagenase (Sigma type 1; 0.5 mg ml^{-1})²⁷ and DNase (Sigma type 1; $13\text{ }\mu\text{g ml}^{-1}$) in a Ca^{2+} - and Mg^{2+} -free Locke's buffer²⁸ and purified by fractionation on a self-generating gradient of Percoll (Pharmacia) as described previously^{19,29}. By this procedure 195×10^6 chromaffin cells of density $1.060.01\text{ g ml}^{-1}$ were obtained from three adrenal glands (wet weight of medullae 25.8 g) with an adrenaline:noradrenaline ratio of 3:1. The cells were plated in a medium consisting of 80% Eagle's minimal essential medium (MEM) (Gibco), 10% human placental serum (heat-inactivated), 2% 9-day chick embryo extract (50% in balanced salt solution), 600 mg% glucose, cytosine arabinoside (10^{-5} M), 5-fluorodeoxyuridine (10^{-5} M), uridine (10^{-5} M), penicillin (200 mg ml^{-1}) and streptomycin (100 mg ml^{-1})³⁰, on collagen-coated plastic³¹ tissue culture dishes (Corning, 35 mm, 10^6 chromaffin cells) or Aclar 33C plastic 'hats' (Allied Chemical 15 mm, 5×10^5 chromaffin cells), and incubated in a humid atmosphere at 36°C for 6 days before use. The media were replaced at days 2 and 4; and on days 5 and 6 Bacitracin (Sigma) was added to a final concentration of $5 \times 10^{-5}\text{ M}$. Immunofluorescence staining of the cells (*b*) with the anti-Leu-enkephalin serum (R164B, 1/90) was shown to be specific by its total inhibition when preabsorbed with synthetic Leu-enkephalin (Peninsula)²¹. Furthermore, cultures incubated with preimmune serum or with the fluorescent conjugate alone showed no specific fluorescence²¹. The cells also contained high concentrations of catecholamine in their perikarya and varicose terminals as demonstrated by the formaldehyde-glutaraldehyde ('Faglu')³² fluorescence histochemical technique. The final preparations were observed unmounted (for immunofluorescence, in 0.1 M bicarbonate-buffered glycerol; for catecholamine, dry) by incident fluorescence illumination with $\times 10$ or $\times 20$ dry Fluotar (Wild-Leitz) objectives using a Leitz Ortholux-II fluorescence microscope fitted with a Ploem incident illuminator and dichroic mirror primary and secondary filters optimal for fluorescein isothiocyanate or for catecholamine fluorescence. The results were recorded on Tri-X (Kodak) film rated at 400ASA. Exposure times were in the range 5–10 s.



with this general property, the dose-response curve for release of endogenous catecholamines and enkephalins by nicotine (Fig. 2) showed similar responses with inhibition at high concentrations of agonist. At day 6, the adrenal paraneurons contained 699 ± 51 ($n = 7$) pg Leu-enkephalin per 10^6 cells and 20.7 ± 1.7 ($n = 7$) μg catecholamines per 10^6 cells: the molar ratio of catecholamines to Leu-enkephalin released by $5 \times 10^{-5}\text{ M}$ nicotine (105×10^3) was similar to that present in the cells (94×10^3).

A recent study¹⁷ showing that Met-enkephalin circulates in human plasma as the intact pentapeptide and that its concentration in the adrenal vein is twice that in the internal jugular or in the peripheral circulation, raises the possibility that enkephalins are secreted together with catecholamines from the

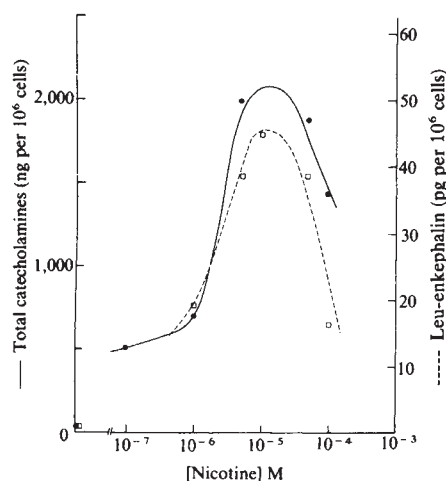


Fig. 2 Dose-response curves for release of endogenous catecholamines and Leu-enkephalin by nicotine. Adrenal paraneurons cultured on 14 Aclar hats were equilibrated for 15 min in KRH medium (containing 2.2 mM Ca^{2+} and $5 \times 10^{-5}\text{ M}$ Bacitracin), and this medium was then replaced by the same medium but containing the concentrations of nicotine indicated. At each concentration of agonist, two hats containing 5×10^5 cells per hat were pooled for analysis. After 5 min at 37°C , the media were removed, acidified and chilled on ice and the cells collected for analysis of endogenous catecholamines and Leu-enkephalin as described in Table 1 legend.

adrenal medulla. This is strongly supported by the parallel release of catecholamines and enkephalins recently demonstrated in perfused dog adrenal¹⁵ and by the present study. Further, the close correspondence of release of adrenaline and Leu-enkephalin (Table 1), and the previous immunohistochemical evidence¹³ for co-storage of noradrenaline with Met-enkephalin in adrenal medullary gland cells may be of physiological significance. There is increasing evidence that amines and peptides may co-exist not only in adrenal chromaffin cells but also in several nekonal systems²⁴. The present demonstration that amines and peptides are released simultaneously from adrenal chromaffin cells in response to a stimulus raises the possibility of co-secretion of catecholamines and enkephalins from adrenergic neurones and the question of the role of this co-secretion.

This study was supported in part by a grant to B.G.L. from the Canadian MRC. J.R. was supported by the Institute National de la Santé et de la Recherche Médicale (France) and D.M.D. by a pre-doctoral fellowship from the Muscular Dystrophy Association of Canada. We thank Mr Joe Donahue for photography, Mrs Gwen Landrigan for typing the manuscript, and Dr R. P. Elde for providing the antisera used for the immunofluorescence studies.

Note added in proof: We have recently shown that Leu- and Met-enkephalin are co-stored within adrenaline (but not within noradrenaline) cells in the bovine adrenal medulla (B.G.L., R. Day, R. P. Elde and P. R. C. Howe, unpublished). A 'preliminary note' by S. M. Stine, H.-Y. T. Yang and E. Costa (*Neuropharmacology* **19**, 683–685 [1980]) indicates that Met-enkephalin-like immunoreactive material is released from isolated bovine chromaffin cells both spontaneously and in response to high concentrations of K^+ (56 mM) and acetylcholine (10^{-4} M).

Received 30 June; accepted 9 October 1980.

- Hughes, J. *et al.* *Nature* **258**, 577–579 (1975).
- Simantov, R. C. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2515–2519 (1976).
- Bloom, F., Battenberg, E., Rossier, J., Ling, N. & Guilleman, R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 1591–1595 (1978).
- Frederickson, R. C. *A. Life Sci.* **21**, 23–42 (1977).
- Bayon, A., Rossier, J., Mauss, A., Bloom, F. E. & Iverson, L. L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 3503–3506 (1978).
- Henderson, G., Hughes, J. & Kosterlitz, H. W. *Nature* **271**, 677–679 (1978).
- Alumets, J., Hakansson, R., Sundler, F. & Chang, K. J. *Histochemistry* **56**, 187–196 (1978).
- Hughes, J., Kosterlitz, H. W. & Smith, T. W. *Br. J. Pharmac.* **61**, 639–647 (1977).
- Schultzberg, M. *et al.* *Acta physiol. scand.* **103**, 475–477 (1978).

10. Schultzberg, M. *et al. Neuroscience* **4**, 249–270 (1979).
11. Di Giulio, A. M. *et al. Neuropharmacology* **17**, 989–992 (1978).
12. Di Giulio, A. M., Yang, H. Y., Fratta, W. & Costa, E. *Nature* **278**, 646–647 (1979).
13. Schultzberg, M. *et al. Neuroscience* **3**, 1169–1186 (1978).
14. Viveros, O. H., Diliberto, E. J., Hazum, F. & Chang, K. J. *Molec. Pharmac.* **16**, 1101–1108 (1979).
15. Viveros, O. H., Diliberto, E. J., Hazum, E. & Chang, K. J. *Psychopharmacologia* **22**, 191–204 (1980).
16. Stern, A. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **76**, 6680–6683 (1979).
17. Clement-Jones, V., Lowry, P. J., Rees, L. H. & Besser, G. M. *Nature* **283**, 295–297 (1980).
18. Livett, B. G., Kozousek, V., Mizobe, F. & Dean, D. M. *Nature* **278**, 256–257 (1979).
19. Mizobe, F., Kozousek, V., Dean, D. M. & Livett, B. G. *Brain Res.* **178**, 555–566 (1979).
20. Lemaire, S., Lemaire, T., Dean, D. M. & Livett, B. G. *Nature* **288**, 303–304 (1980).
21. Livett, B. G. & Dean, D. M. *Neuropeptides* **1**, 3–13 (1980).
22. Rossier, J., Dean, D. M., Livett, B. G. & Udenfriend, S. *Life Sci.* (in the press).
23. Rossier, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 5162–5165 (1977).
24. Hökfelt, T., Johansson, D., Ljungdahl, A., Lundberg, J. M. & Schultzberg, M. *Nature* **284**, 515–521 (1980).
25. Miura, Y., Campese, V., De Quatro, V. & Meijer, D. J. *Lab. clin. Med.* **89**, 421–427 (1977).
26. Renzini, V., Brunori, C. A. & Valori, C. *Clin. chim. Acta* **30**, 587–594 (1970).
27. Fenwick, E. M., Fajdiga, P. B., Howe, N. B. S. & Livett, B. G. *J. Cell Biol.* **76**, 12–30 (1978).
28. Trifaró, J. M., Poisner, A. M. & Douglas, W. W. *Biochem. Pharmac.* **16**, 2095–2100 (1967).
29. Dean, D. M., Watson, L., Bray, G. M. & Livett, B. G. *Neurotoxins* (eds Chubb, I. W. & Geffen, L. B.) 261 (Adelaide University Union Press, 1979).
30. Wood, P. M. *Brain Res.* **115**, 361–375 (1976).
31. Bornstein, M. B. *Lab. Invest.* **7**, 134–137 (1958).
32. Furness, J. B., Costa, M. & Blessing, W. W. *Histochem. J.* **9**, 745–750 (1977).

Photolabelling of cholera toxin subunits during membrane penetration

Bernadine J. Wisnieski & John S. Bramhall*

Department of Microbiology and The Molecular Biology Institute,
University of California, Los Angeles, California 90024

There has been much speculation about the mechanism by which cholera toxin exerts its effect on the cytoplasmic side of the membranes with which it interacts^{1–4}. After the pentamer of B subunits (5B) binds to membrane receptors, particularly the monosialylganglioside GM₁, the disulphide-linked dimer A₁SSA₂ (which together with 5B constitutes the complete toxin) is thought to penetrate the membrane, perhaps through a channel formed by 5B and become reduced so that A₁SH units reach the cytoplasm and stimulate adenylate cyclase. Evidence for this mechanism is circumstantial^{1,4,5}. If it is correct, a compound which will specifically label intramembranous sections of the toxin should label the channel-forming B subunits but not the channel-contained A₁ subunit. We have tested this prediction with a photoreactive glycolipid compound^{6,7} and have obtained the opposite result. Therefore, we propose that only the A₁ subunit enters the membrane and we provide here data on the kinetics of that process.

The experimental protocol described is a direct and non-perturbing method for studying the dynamics of membrane penetration by proteins and other molecules. For simplicity, we used the Newcastle disease virus (NDV) system, earlier characterized with the photoreactive glycolipid probe⁶, as a membrane target for cholera toxin because the viral envelope contains only three proteins, designated HN, F and M (ref. 8); surface neuraminidase activity resident in the HN protein makes NDV especially rich in GM₁ (ref. 8). The glycolipid probe 12-(4-azido-2-nitrophenoxyl)-stearoyl[1-¹⁴C]glucosamine (12APS-GlcN) is unique in that it will spontaneously insert into any membrane, and studies with a spin-labelled (nitroxide) counterpart suggest that the glucosamine moiety restricts it to the surface monolayer for an appreciable time after insertion⁹. The photoreactive group of 12APS-GlcN is located on the stearic acid chain of the amphiphilic compound. It reacts with vicinal compounds only when samples are exposed to UV light. Photolabelling is relatively nonspecific⁷. The probe labels coliphage M13(f₁) coat protein only after incorporation into the bilayer of

artificial vesicles⁷. Further, and most significantly, it labels only those residues of the protein which reside within the outer monolayer (ref. 10 and unpublished data). Chowdry and Westheimer¹¹ have reviewed membrane photolabelling studies. We have previously shown that cholera toxin can be photolabelled after binding to NDV charged with probe^{12,13}. Here we analyse the kinetics of specific subunit insertion into the membrane.

Figure 1 shows the separation of proteins derived from cholera toxin, NDV and NDV with bound cholera toxin. The difference between the amount of toxin bound to NDV and the total toxin present (60 µg per sample) can be estimated by comparing lanes 1 and 3 and lanes 4 and 5. Typically, 3–5 µg of the toxin bound to NDV in the conditions used. Next to each stained gel the corresponding fluorogram shows proteins radiolabelled by the photoreactive probe (Fig. 1). Irradiation for 15 s allowed as much labelling of membrane proteins as irradiation for 6 min (data not shown); thus we conclude that most of the probe was photoactivated. Gel bands designated VP represent viral proteins whose photolabelling has been described elsewhere⁶. About 15–25% of the protein in band

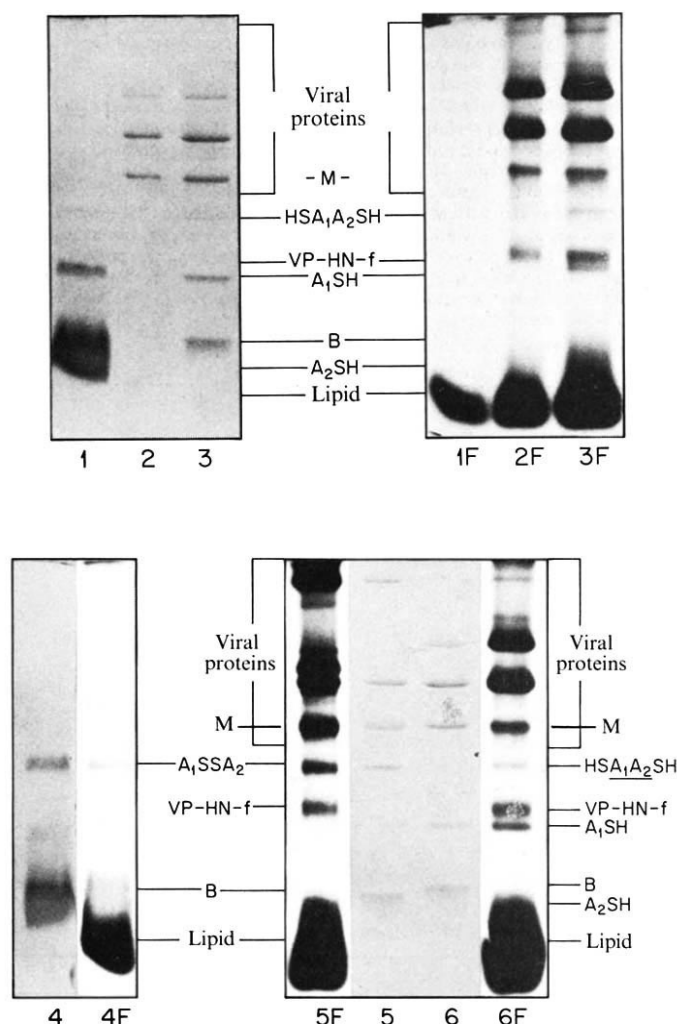


Fig. 1 Radiolabelling of NDV and cholera toxin proteins with the photoreactive probe 12APS-GlcN. Stained gel lanes are 1–6; corresponding fluorogram lanes are 1F–6F. Unreduced samples are in lanes 4 and 5. Lanes 1 and 4 contain cholera toxin (60 µg) labelled in solution and acid precipitated; lane 2 contains NDV (30 µg protein) labelled in suspension and pelleted. Lanes 3, 5, and 6 contain cholera toxin labelled after attachment to NDV for 1 min at 37 °C. Each sample was irradiated at 360 nm for 15 s. The amount of probe (50 mCi mmol⁻¹) per sample was 4 × 10⁻² µCi. Total equilibration time with probe was 16 min at 37 °C for toxin alone and for NDV alone, and 15 min for NDV to which toxin was added 1 min before irradiation. Procedures as described in Table 1 legend.

* Present address: Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305.

Table 1 Photoreactive radiolabelling of cholera toxin subunits and viral proteins as a function of time and temperature of incubation

Unreduced protein bands	Net c.p.m.						NDV alone	Toxin alone
	0 °C, 15 min	37 °C, 30 s	37 °C, 1 min	37 °C, 3 min	37 °C, 15 min			
VP-M	209	174	196	210	192	205	0	
A ₁ SSA ₂	6	91	136	86	71	0	21	
VP-HN-fragment	100	100	105	99	100	102	0	
Total c.p.m.	315	365	437	395	363	307	21	

Reduced protein bands	Net c.p.m.								NDV alone	Toxin alone
	0 °C, 15 min	37 °C, 30 s	37 °C, 1 min	37 °C, 2 min	37 °C, 3 min	37 °C, 10 min	37 °C, 15 min			
VP-M	200	194	191	223	208	195	190	201	0	
HSA ₁ A ₂ SH	2	23	35	27	23	20	19	0	4	
VP-HN-fragment	109	108	101	107	110	103	99	102	0	
A ₁ SH	5	69	107	84	71	56	45	0	16	
Total c.p.m.	316	394	434	441	412	374	353	303	20	
A ₁ SH + HSA ₁ A ₂ SH	7	91	142	111	94	96	64	0	20	

Experiments were carried out under red safety lights until completion of electrophoresis to avoid activation of probe in unirradiated controls. Cholera toxin, purified¹⁴ and stored at 6 mg ml⁻¹ in 0.1 M sodium phosphate buffer, pH 7, was thawed and diluted to 1 mg ml⁻¹ with 5 mM sodium phosphate-buffered saline containing 1 mM EDTA, pH 7.4 (PBSE). Two aliquots (0.6 ml) were spun at 149,000g for 30 min to remove any aggregates. The supernatant was used throughout. NDV strain HP-16, isolated as described elsewhere⁶, was diluted to 450 µg protein per 9.4 ml PBS. The photoreactive probe 12APS-GlcN (50 mCi mmol⁻¹), synthesized as described elsewhere¹⁵, was diluted to 90,000 c.p.m. per 10 µl of ethanol. In tube A, 150 µl of probe was dried and 50 µl of ethanol added and in tube B, 40 µl of probe was dried and 13 µl of ethanol added. The NDV suspension (9.4 ml) was added to tube A and 240 µl of toxin solution plus 2.5 ml PBSE added to tube B. Both tubes were incubated at 37 °C for 15 min. During this time, toxin solution was dispensed in 60-µl portions into 11 borosilicate tubes (6 × 50 mm), then 0.6 ml of NDV from tube A was added to each of these tubes, which were incubated for periods of time from 0.5 to 15 min before irradiation for 15 s at 366 nm with a mineral lamp (UV-Products, San Gabriel, California). Usually two samples were irradiated at each time point to provide reduced and non-reduced samples for gels. One unirradiated sample provided a control for background radioactivity. Two samples (0.6 ml) of NDV from tube A were incubated for an additional 15 min at 0 °C and at 37 °C before irradiation. Another two 0.6 ml samples of NDV were cooled to 0 °C before being added to two small tubes, each of which contained 60 µl of chilled toxin solution. One of these was irradiated after 1 min at 0 °C, the other after 15 min at 0 °C. The contents of tube B were divided into four 0.6-ml samples which were incubated at 0 °C for 1 min, 0 °C (15 min), 37 °C (1 min) and 37 °C for 15 min before irradiation. The entire experiment was repeated to provide a duplicate set of samples for analysis. Each time, 60-µl portions of toxin solution were added to 0.6 ml of PBSE and incubated for 30–60 min at 37 °C and centrifuged to determine how much toxin aggregated and could be pelleted without acid precipitation. This routine check was performed because it was found that commercial lyophilized toxin did not give reproducible results when used as a substitute for freshly isolated toxin (probably because it proved to be extremely difficult to completely solubilize). All samples were pelleted at 149,000g for 30 min on a Beckman SW50.1 rotor in buckets fitted with 0.7 ml adaptors. To each sample containing toxin with probe alone (from tube B), 50% trichloroacetic acid (60 µl) was added after irradiation and the samples cooled to 0 °C before centrifugation. Pellets were boiled for 2 min in 40 µl of reducing or non-reducing SDS-buffer¹⁶. Electrophoresis and fluorography were carried out as described elsewhere¹³. Gel slices were oxidized to ¹⁴CO₂, which was collected and counted⁶. Top, data from non-reduced samples; bottom, reduced samples. Columns designated NDV alone and toxin alone refer to samples incubated independently with probe for 15 min at 37 °C, before irradiation. The sample of toxin alone (60 µg) was acid precipitated. The other columns represent components of toxin which pelleted with virus, estimated to be 5% of the 60 µg added. VP, viral protein; HN-f, HN-fragment. The HSA₁A₂SH peptide is the reduced but uncleaved A₁A₂ peptide. Normally A₁SSA₂ completely reduces to A₁SH and A₂SH; however, in rapidly isolated cholera toxin preparations, not all the linear A₁A₂ peptide is nicked between the two cysteines which form the disulphide bridge between A₁ and A₂.

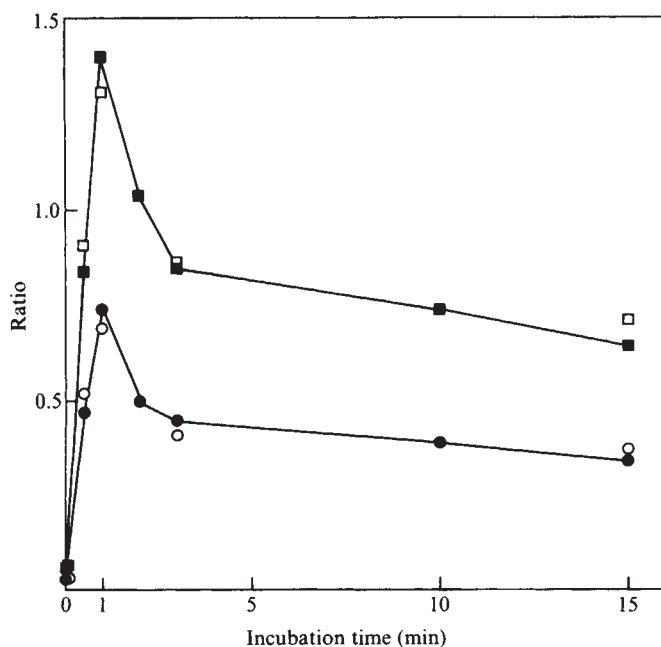
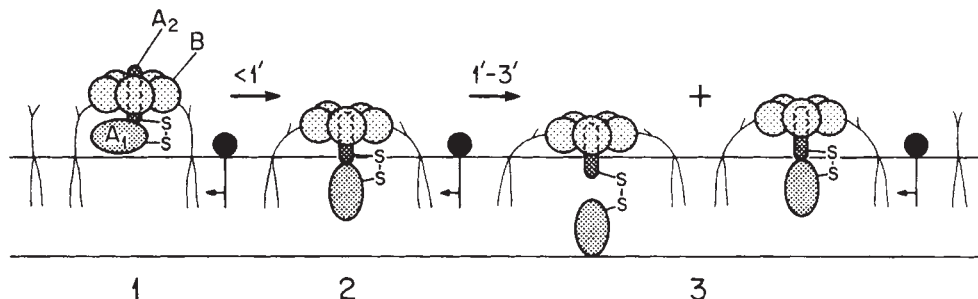


Fig. 2 The kinetics of photoreactive labelling of cholera toxin A subunits. Open symbols, ratios calculated from Table 1 (top); solid symbols, ratios calculated from Table 1 (bottom). Zero time points represent ratios calculated from 0°, 15' samples in Table 1. Solid symbols, ratio of radiolabel in A₁SH + HSA₁A₂SH (unnicked) to radiolabel in viral protein M (●) and HN-f (■). Open symbols, ratio of radiolabel in A₁SSA₂ (nicked plus unnicked) to radiolabel in viral protein M (○) and HN-f (□). The ratio of radiolabel in HN-f to radiolabel in M was relatively constant (average 0.51; range 0.47–0.57).

A₁SSA₂ represents A₁A₂ linear peptide which was not proteolytically cleaved during toxin purification¹² (the underlining of A₁A₂ is meant to distinguish this linear (uncleaved) peptide from the cleaved peptide A₁A₂). The rest of the band represents peptide which was cleaved between the disulphide bridge into discrete A₁ and A₂ subunits. The B subunit contains an internal disulphide bond and migrates with a larger apparent molecular weight when reduced. Reduced toxin samples show a faint band representing the reduced, but uncleaved, linear peptide HSA₁A₂SH (ref. 12), as well as A₁SH and A₂SH. The A₂SH subunit does not stain effectively. It migrates ahead of B and can be detected when ¹²⁵I-labelled toxin is electrophoresed¹². In the fluorograms shown, the A₂SH peptide could not be resolved with any confidence from the diffuse band of radiolabelled lipids.

Other samples, including a series irradiated at defined times after the addition of toxin to NDV, were similarly electrophoresed. Bands representing viral proteins VP-M and VP-HN-fragment and toxin subunits (except A₂SH) were cut from stained gels after fluorography (because VP-HN-fragment does not stain (Fig. 1)), and oxidized to ¹⁴CO₂ which was trapped and counted. The level of radioactivity in the B subunit was not significantly above background in conditions tested (Fig. 1 and ref. 13). As previously demonstrated^{6,13}, unirradiated samples did not contain any radiolabelled proteins. Radioactivities of the pertinent bands are shown in Table 1. The labelling of A₁ plus A₂, that is, the net radioactivity associated with unreduced A₁SSA₂ (cleaved plus uncleaved), is expressed as a function of time and temperature of incubation with NDV before the 15 s irradiation. To standardize the data between gel lanes, raw data were later expressed as ratios within a particular lane. Radiolabelling of the two viral proteins chosen for ratio calculations did not vary with time or temperature or the presence of reducing agent, and therefore accurately reflect the amount of sample in each gel lane.

Fig. 3 Transmembrane kinetics of the active A_1 subunit of cholera toxin. Step 1, B subunits bind to ganglioside receptors (forked structures). Within 1 min of binding at 37 °C, A_1 insertion into the outer monolayer of the membrane is complete (Step 2). Within 2 min of insertion, A_1 equilibrates between the outer and inner monolayers (Step 3). The equilibrium achieved is undoubtedly a dynamic one; however, relative to the kinetics of the photolabelling reaction, the position of each A_1 is fixed. The small arrow represents the photoreactive group of 12APS-GlcN.



Data in Table 1 indicate that the A subunit of toxin can enter the vicinity of photogenerated reactive groups within seconds of binding to the viral membrane. To assess whether the radioactivity in the A_1SSA_2 band was due to labelling of A_1 , A_2 , or both, the ratios of counts in this band to the counts in both VP-M and VP-HN-f were plotted as a function of incubation time at 37 °C with NDV (Fig. 2, open symbols). The counts in A_1SH and in uncleaved HSA_1A_2SH were summed and expressed as a ratio to counts in VP-M, and to counts in VP-HN-f (Fig. 2, solid symbols). Surprisingly, Fig. 2 shows that the ratios obtained as a function of time using total A_1SSA_2 (unreduced) counts and the ratios obtained using a formula in which 75–85% of A_2 is not included (due to reduction and dissociation of the cleaved fraction of A_1SSA_2 molecules) are not significantly different. From this we conclude that the majority of the radiolabel associated with the A_1SSA_2 band (unreduced) and the SHA_1A_2SH (reduced, uncleaved) is actually attached to the A_1 subunit of the toxin and not the A_2 portion.

The most striking feature of the data presented in Table 1 and in Fig. 2 is that the A component of toxin, more specifically the A_1 portion (from the rationale above), becomes increasingly accessible to photolabel within the first minute of incubation at 37 °C with NDV. During the next 2 min of incubation, radiolabelling of A_1 decreases by roughly 40% (34–41%). Between 3 and 15 min of incubation, very little change occurs. The accessibility of A_1 to probe after 15 min is 50% (46–54%) of that observed at 1 min. The rapid penetration and labelling of A_1 resemble that of complement component C9 on addition to vesicles containing C5b-8 (refs 17, 18 and unpublished data). The ratio of label in uncleaved toxin (HSA_1A_2SH) to label in A_1SH (calculated from data in Table 1) undergoes a slight increase with time; the significance of this remains to be determined. The kinetics of labelling of the A_1 subunit was verified qualitatively by exposing X-ray film to gels for various time periods before development. The first A_1 -containing bands to produce silver grains in the film emulsion were always from the very early incubation times. Little or no labelling of any toxin subunit occurred when toxin and NDV were incubated together at 0 °C before irradiation, regardless of incubation time, even though NDV bound the same amount of toxin at this temperature as at 37 °C and displayed no change in its own labelling profile.

Values for the extent of labelling of the viral proteins and of the bound cholera toxin A_1 subunit (at the 1 min time point) were all at the level of 4 mol%. A similar value was obtained with M13 coat protein in vesicles⁷. This strongly implies that within 1 min of toxin addition and binding, most of the A_1 associated with virus entered the membrane envelope. If we assume that only 10% or 1% of the virally bound A_1 entered the membrane, the calculated level of A_1 -specific labelling needs to be increased to 40 and 400 mol%, respectively, an unlikely occurrence unless an equivalent level of preference of probe for A_1 is proposed. Thus, we propose that most of the A_1 associating with virus actually penetrates the bilayer. We believe this to be the first such demonstration.

Our data are incompatible with the Gill¹ model for toxin entry because if the B pentamer formed a transmembrane channel, we would expect to label B but not A (which would be protected

from the probe by residues of the B channel). The reverse was actually observed, and the detected entry of the enzymatically active A_1 subunit was found to be temperature-dependent, occurring at 37 °C (a temperature compatible with physiological activity), but not at 0 °C (a temperature compatible with binding but not activity).

Explanation of the kinetic data shown in Fig. 2 must answer two questions: (1) where does 50% of the A_1 entering the membrane go and (2) why does only 50% of the entering A_1 leave? One proposal (see Fig. 3), is based on the assumption that the probe remains restricted to the outer monolayer. This is a reasonable assumption because, as previously mentioned, all available data are consistent with an asymmetric distribution of such a compound (refs 9, 10 and unpublished data). Within 1 min of toxin binding, the A_1 subunit penetrates the outer monolayer of the membrane (step 1). The kinetics of A_1 entry between 0 and 1 min would reflect the kinetics of toxin binding to receptor¹⁹ and subunit penetration. Between 1 and 3 min, the rapid decrease in A_1 labelling suggests that about half of the A_1 molecules (or one half of each molecule) leave the vicinity of probe within 2 min of entry (step 2). We visualize this as an equilibration of A_1 between the outer and inner monolayers until roughly one half is in each monolayer. The calculated half-time for the A_1 equilibration process (decrease in labelling) is of the order of 10–20 s. This time factor is not unreasonable for a diffusion-limited process in a viscous medium. At any time after 3 min, only 50% of the A_1 that entered the membrane remains accessible to probe, indicating very little change in A_1 distribution after 3 min (step 3).

By use of photoreactive probes to investigate the dynamics of toxin translocation in other systems, we can start to examine the specific roles of membrane composition, fluidity and asymmetry on protein traversal through membranes.

B.J.W. is the recipient of USPHS Research Career Development Award GM-00228. J.S.B. was a Fulbright-Hayes Postdoctoral Scholar. Research was supported by USPHS Grant GM-22240, the University of California Cancer Research Coordinating Committee and the Academic Senate.

Received 7 July; accepted 10 November 1980.

- Gill, D. M. *Biochemistry* **15**, 1242–1248 (1976).
- Sahyoun, N. & Cuatrecasas, P. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3438–3442 (1975).
- van Heyningen, S. *Biochem. J.* **168**, 457–463 (1977).
- Tosteson, M. T. & Tosteson, D. C. *Nature* **275**, 142–144 (1978).
- Moss, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **73**, 3480–3483 (1976).
- Bramhall, J. S., Shiflett, M. A. & Wisnieski, B. J. *Biochem. J.* **177**, 765–768 (1979).
- Hu, V. W. & Wisnieski, B. J. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5460–5464 (1979).
- Scheid, R. & Choppin, P. W. *Virology* **57**, 475–490 (1974).
- Wisnieski, B. J. & Iwata, K. K. *Biochemistry* **16**, 1321–1326 (1977).
- Simon, P. & Wisnieski, B. J. *Fedn Proc.* **39**, 2190 (1980).
- Chowdry, V. & Westheimer, F. H. A. *Rev. Biochem.* **48**, 293–320 (1979).
- Wisnieski, B. J., Shiflett, M. A., Mekalanos, J. & Bramhall, J. S. *J. supramolec. Struct.* **10**, 191–197 (1979).
- Wisnieski, B. J. & Bramhall, J. S. *Biochem. biophys. Res. Commun.* **87**, 308–313 (1979).
- Mekalanos, J. J., Collier, R. J. & Romig, W. R. *Infect. Immun.* **20**, 552–558 (1978).
- Bramhall, J. S., Ishida, B. & Wisnieski, B. J. *J. supramolec. Struct.* **9**, 399–406 (1978).
- O'Farrell, P. H. *J. biol. Chem.* **250**, 4007–4021 (1975).
- Hu, V. W., Esser, A. F., Podack, E. R. & Wisnieski, B. J. *Fedn Proc.* **39**, 1971 (1980).
- Hu, V. W., Esser, A. F., Podack, E. R. & Wisnieski, B. J. *Proc. 4th int. Congr. Immun. Abstr.* No. 15.2.07 (1980).
- Cuatrecasas, P. (ed.) *The Specificity and Action of Animal, Bacterial and Plant Toxins* (Chapman & Hall, London, 1977).

The magnitude of signal amplification by ligand-induced receptor clustering

Charles DeLisi

Laboratory of Theoretical Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Lateral mobility of plasma-membrane proteins is now documented for a variety of cell systems. Evidence is accumulating that the molecular clustering allowed by such mobility may be important in the transduction of information at the cell surface¹⁻⁷. Moreover, in a wide variety of systems, only a very small fraction of receptors need to be bound to induce a response^{8,9}, such as in the mast cell/basophil system where receptor clusters as small as dimers can induce a response¹⁰ and only three or four such dimers are necessary for local degranulation¹¹. As receptors are mobile, some clustering is expected even in the absence of ligand. As an approximate indication of the numbers involved, a consideration of constraints on translational entropy by spontaneous pair formation predicts¹² about 100 pairs on average for a cell with 500,000 receptors (typical of mast cells¹³), if a pair is defined as two receptors whose peripheries are within 5 Å. This estimate need not present a problem as it can be reduced by several orders of magnitude by postulating orientational requirements, repulsive potentials and/or more stringent constraints on translational entropy. Moreover, some cell preparations do show a slow Ca²⁺-dependent background release² and this may, in fact, reflect spontaneous clustering and suggest that the control of activity is not stringent for those cells. An alternative view of the regulation of activity, however, which may have broad implications, is that receptors must be in proximity for some minimum amount of time for the transmission of biological information. This residency requirement may, for example, reflect the time required for the initiation of Ca²⁺ flow¹⁴ or for receptors to change conformation¹⁵ or for the pair to bind an integral membrane protein¹⁶. Whatever the origin, the quantitative predictions of the idea, which I develop here, suggest, for biologically relevant parameter values, an exceedingly sensitive control mechanism that can amplify the probability of an event by a factor of 10²⁰ or more.

An estimate of the probability that a pair, having formed, will be active t s later is easily made if the pair separation times are exponentially distributed. Thus, if t_- is the mean time for a pair to separate by diffusion to some distance Δ beyond which it cannot transmit a signal, the fraction of pairs remaining within Δ for t s after pair formation is $\exp(-t/t_-)$. The fraction that remains within Δ long enough to transmit a signal is approximately

$$\nu_0 = \exp(-t_D/t_-) \quad (1)$$

where t_D is the minimum time the perimeters must be within Δ for transduction of a signal.

When receptors are cross-linked by ligand, pair separation will depend not just on diffusion but on the time required to break the ligand-receptor bond. If k_1 and k_{-1} are the reactive forward and reverse rate constants for ligand-receptor interaction respectively, then the effective rate constant for pair dissociation is^{17,18}

$$k_r = \frac{k_-k_{-1}}{k_1 + k_-} \quad (2)$$

and the effective dissociation time, t_r , is

$$t_r = 1/k_r \quad (3)$$

In equation (2), the diffusive reverse rate constant, k_- , is related to the diffusive dissociation time by

$$k_- = 1/t_- \quad (4)$$

For diffusion-limited reactions ($k_1 \gg k_-$), equation (2) becomes

$$k_r = k_-/K_0$$

where

$$K_0 = k_1/k_{-1} \quad (5)$$

is the intrinsic equilibrium constant of a ligand site for a receptor site. The ligand-induced amplification (A) of the transduction probability is defined as the ratio of the probability of transduction in the presence of ligand to that in its absence. Thus

$$A = \exp \left[-\frac{t_D}{t_-} \left(\frac{1}{K_0} - 1 \right) \right] \quad (6)$$

Note that equation (6) is the ratio of two probabilities, each probability being obtained by division by the actual number of pairs. Hence the actual number of pairs does not enter equation (6). Had a different definition of amplification been adopted in terms of an increase in numbers of pairs (and not just their duration), the amplification would be somewhat larger than estimated below, by perhaps two orders of magnitude. It will become evident that the distinction between the two definitions is neither qualitatively nor quantitatively important. Equation (6) was chosen because of its relative simplicity.

K_0 will be large even if binding is weak. For example, a binding free-energy change of 5.5 kcal corresponds to an intrinsic equilibrium constant of 10⁴ at room temperature. A plot of amplification as a function of the ratio t_D/t_- for $K_0 = 10^4$ rises exponentially, reaching 10¹⁰ at a ratio of 25 and 10³² at a ratio of 75 (Fig. 1). Thus the transduction probability can be changed from essentially zero in the absence of ligand (for example, $\approx 10^{-10}$ at $t_D/t_- = 25$) to close to one in its presence. The large amplifications are consequences of very low levels of spontaneous signal transmission in the absence of ligand.

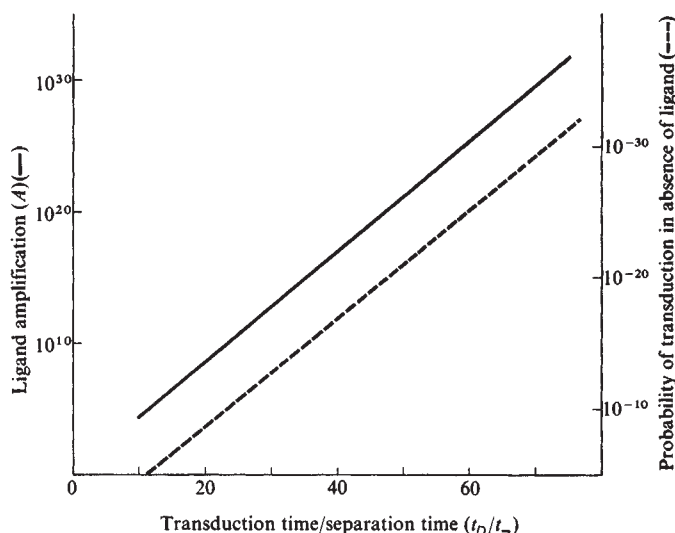


Fig. 1 Probability of transduction in the absence of ligand (---) and the amplification achieved (—) with a low-affinity ligand having an intrinsic equilibrium constant for the receptor of $K_0 = 10^4$.

Table 1 Mean time for a receptor pair to separate to a distance Δ

$\Delta(\text{\AA})$	95	100	105	110	120	130
$t_{-}(\text{s})$	1.3×10^{-6}	5.3×10^{-6}	1.2×10^{-5}	1.7×10^{-5}	4.3×10^{-5}	7.4×10^{-5}

Diffusion coefficient = $3 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$, distance of closest approach = 90 $\text{\AA}$.

How reasonable is the range of ratios for t_D/t_{-} shown in Fig. 1? Transduction times are expected to be very rapid. For example, conformational changes in proteins in solution generally occur in 10^{-2} – 10^{-3} s (ref. 19), though such changes might take somewhat longer in a membrane. Consequently, if transduction requires a conformational change, t_D would be of the order of milliseconds or longer. Similarly, experiments in which the flow of Ca^{2+} into a cell was followed indicate that uptake is 90% complete within 30 s, suggesting a time constant of seconds or less. Thus a transduction times of about 10^{-3} s would seem to be a reasonable lower bound. Amplification would then, according to Fig. 1, require diffusive dissociation times of $\approx 10^{-4}$ s.

The diffusive dissociation time can be estimated as follows. Consider circular receptor disks of diameter s with the plane of the disks coinciding with the plane of the membrane. The coordinate origin is fixed at the centre of one of the disks. Then, if the centre of a second disk is at $s \leq \rho \leq s + \Delta$, the mean time $W(\rho)$ required for its first arrival at $s + \Delta$ satisfies²⁰

$$D \nabla^2 W(\rho) = -1 \quad (7)$$

where D is the sum of the diffusion coefficients of the disks. The solution to equation (7) subject to the boundary conditions

$$W(s + \Delta) = 0$$

$$\left(\frac{dW}{d\rho} \right)_{\rho=s} = 0$$

is

$$W(\rho) = \frac{(s + \Delta)^2 - s^2}{4D} + \frac{s^2}{2D} \ln(s/(s + \Delta)) \quad (8)$$

The mean time for a particle in the active region to escape is obtained by averaging equation (8) over all distances between s and $s + \Delta$. Thus,

$$t_{-} = \frac{2}{(s + \Delta)^2 - s^2} \int_s^{s + \Delta} \rho W(\rho) d\rho \quad (9)$$

Diffusive dissociation times were calculated according to equation (9) as a function of Δ (Table 1), using a receptor diameter of 90 $\text{\AA}$ and a diffusion coefficient of $3 \times 10^{-10} \text{ cm}^2 \text{ s}^{-1}$, numbers typical of the mast cell system^{21,22}. The calculation is not of course intended to be precise, but is presented here only to provide perspective for evaluating the plausibility of the range of ratios in Fig. 1.

The above analysis assumes that when receptors are cross-linked, they are always within the active radius. However, this assumption is not compatible with molecular flexibility. Suppose, for example, that the doubly bound ligand were fully flexible and its maximum extension exceeded Δ . Then within the framework of the residency-time requirement, the ligand would not be sensed by the cell and the amplification would be one. The reason is that if the ligand is so flexible that it does not affect the diffusion of one receptor relative to its partner, the time to escape from the active region will not be changed; only the rate of entry into the region will change. A fully flexible molecule is, however, unrealistic.

For molecules in solution, some fluctuation in distances between groups is expected, even if the intervening chain is short. However, when both groups are constrained to lie on a cell surface as a result of bonding to receptors, the ability of the intervening backbone bonds to rotate is severely restricted—a fact easily verified by examination of molecular models. The chain is then effectively frozen in whatever configuration it was in when the second bond formed. From this point of view, the distances between reactive groups on a ligand, averaged over the collection of multiply bound ligands, will reflect the distribution of distances between groups when the ligand is free, even though the distance between groups on any particular molecule does not fluctuate. But only that fraction of multiply bound ligands having configurations constraining the peripheries of the receptors to be within Δ will be recognized. Thus the reduction in the concentration of active pairs, at a fixed ligand concentration, will be the main effect of flexibility and the amplification factor given by equation (6) remains essentially unchanged. Experiments using stiff chains or using flexible chains whose dimensions have been determined independently, for example by energy transfer, may elucidate the validity of these remarks.

Finally, note that the form of equation (6) is not necessarily limited to systems that rely on clustering. For example, if the step subsequent to binding were a conformational change, then t_{-} could be interpreted as the natural lifetime of the active conformation in the absence of ligand. In the presence of ligand, the lifetime would be k_{-} , the dissociation time of the ligand. The amplification would then be $\exp[k_{-} - k_{-}t_D] = \exp(k_{-}t_D)$, and the fraction of receptors remaining active for t_D , $\exp(-k_{-}t_D)$. As long as $k_{-}t_D$ is much less than one, changes in k_{-} will not affect the fraction of bound ligands that are active. As $k_{-}t_D$ becomes greater than one, however, activity should drop rapidly. Somewhat similar considerations²³ were used to explain the precipitous drop in cyclic AMP production as the affinity of hyponeurophysal hormones for their receptors decreased, while the total bound concentration remained constant.

In conclusion, the molecular complex mediating information transfer, whether it be a receptor cluster or a particular receptor conformation, is assumed to be present at all times, but intrinsically unstable with a decay time that is short compared with the time required for information transfer. Signals in these circumstances are too low to be distinguished from background noise. Ligands, by stabilizing an active complex so that its decay time exceeds the time required for information transfer, selectively amplify particular signals. Numerical estimates indicate that such amplification can be very large.

I thank Drs Henry Metzger and Alan Perelson for reading and commenting on the manuscript.

Received 14 July; accepted 10 November 1980.

1. Siraganian, R. P., Hook, W. A. & Levin, B. B. *Immunochemistry* **12**, 149–157 (1975).
2. DeLisi, C. & Siraganian, R. P. *J. Immun.* **122**, 2286 (1979).
3. Dembo, M., Goldstein, B., Sobotka, A. K. & Lichtenstein, L. M. *J. Immun.* **122**, 518 (1979).
4. Fanger, M. W., Hart, D. A., Wells, J. V. & Nisonoff, A. *J. Immun.* **105**, 1484 (1970).
5. Archer, B. G. & Krakauer, H. *Biochemistry* **16** (1977).
6. Schechter, Y., Hernaez, L., Schlessinger, J. & Cuatrecasas, P. *Nature* **278**, 835 (1979).
7. Kahn, C. R., Baird, K. L., Jarrett, D. B. & Flier, J. S. *Proc. natn. Acad. Sci. U.S.A.* **75**, 4209 (1978).
8. Birnbaumer, L., Pohl, S. L. & Kaumann, A. J. *Adv. Cyclic Nucleotide Res.* **4** (1975).
9. Schlessinger, J. in *Physical Chemistry of Cell Surface Events in Cellular Regulation* (eds Delisi, C. & Blumenthal, R.) (Elsevier, Amsterdam, 1979).
10. Segal, D. M., Taugo, J. & Metzger, H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2993 (1977).
11. DeLisi, C. & Siraganian, R. *J. Immun.* **122**, 2281 (1979).
12. DeLisi, C. *Q. Rev. Biophys.* (in the press).
13. Metzger, H. & Bach, M. K. in *Immediate Hypersensitivity* (ed. Bach, M. K.) 561 (Dekker, New York, 1978).
14. Forman, J. C., Hallet, M. B. & Morgar, J. L. *J. Physiol., Lond.* **271**, 193 (1977).
15. Hiedman, T. & Changeux, J. P. *A. Rev. Biochem.* **47**, 317 (1978).
16. Bourguignon, L. Y. W. & Singer, S. J. *Proc. natn. Acad. Sci. U.S.A.* **74**, 5131 (1977).
17. Eigen, M. in *Quantum Statistical Mechanics in the Natural Sciences* (eds S. L. Minz, & Weidmayer, S. M.) (Plenum, New York, 1974).
18. Bell, G. I. *Science* **200**, 618 (1978).
19. Hammes, G. *Adv. Protein Chem.* **23**, 1 (1968).
20. Berg, H. C. & Purcell, E. M. *Biophys. J.* **20**, 193 (1977).
21. Metzger, H. *Immun. Rev.* **41**, 186 (1978).
22. Schlessinger, J., Webb, W., Elson, E. L. & Metzger, H. *Nature* **264**, 550 (1976).
23. Bergman, R. & Hechter, O. *J. Biol. Chem.* **253**, 3238 (1978).

Evidence for a divided genome in bean golden mosaic virus, a geminivirus

S. Haber, M. Ikegami, N. B. Bajet
& Robert M. Goodman

Department of Plant Pathology, University of Illinois, Urbana,
Illinois 61801

Geminiviruses^{1,2}, a recently discovered group of plant viruses, are the only viruses containing circular single-stranded DNA (ssDNA) found in eukaryotic organisms. Their ssDNA, which is less than half the size (molecular weight (M_r) $7-8 \times 10^5$) (refs 3-6) of that of other ssDNA viruses^{7,8}, is the smallest yet found in autonomously replicating viruses. Geminivirus DNA is encapsidated in paired isometric particles whose monomer units measure ~ 18 nm. This unique capsid morphology was accounted for by an early hypothesis of a functionally divided genome. Among the RNA plant viruses, there are several groups that carry necessary genetic information on more than one nucleic acid species. In the case of geminiviruses, two nucleic acid components were postulated to be carried in each paired particle^{9,10}. Analyses of the physical composition of geminiviruses showed, however, that there is only one DNA molecule in each paired particle^{6,11,12}. We have now investigated the DNA of a geminivirus using restriction endonucleases and a biological assay of infectivity and report here evidence for a divided genome in bean golden mosaic virus (BGMV). Two different nucleotide sequences are found and both seem to be required for infectivity.

BGMV was maintained by sap transmission in *Phaseolus vulgaris* L. ('Top Crop') kept in simulated humid tropical conditions in a growth chamber^{11,13} and purified as described elsewhere^{13,14}. Viral DNA labelled with ³²P *in vivo* was obtained by collecting systemically infected trifoliolate leaves from plants 5 days after inoculation of primary leaves and feeding with ³²P-orthophosphate (1 mCi per leaf) through the cut ends of petioles. The leaves were then maintained for 4 days in a Petri dish by feeding a balanced inorganic salt solution through the petioles.

BGMV DNA is resolved by polyacrylamide gel electrophoresis in 7 M urea into two components which comprise a covalently closed circular molecule (M_r 8×10^5) and a slightly shorter linear molecule⁵; previous evidence suggests that the linear molecule is derived from (or possibly is a precursor of) the circular form¹¹. We separated ³²P-labelled circular and linear molecules by gel electrophoresis and recovered the DNA by electro-elution¹⁵ from excised gel slices. The DNA was then digested separately with restriction endonucleases *HhaI* and *HaeIII* which have sequence-specific activity on the ssDNA of bacterial viruses^{16,17}. DNA from Φ X174 digested with *HaeIII* was used as a standard.

Digestion of linear or circular BGMV DNA with *HhaI* yielded fragments of the same size and relative electrophoretic mobility (data not shown), confirming our previous conclusion that circular and linear forms of BGMV DNA contain the same nucleotide sequences¹¹. Calculation of the length of each BGMV DNA fragment present in complete *HhaI* digests of circular BGMV DNA revealed that the total length of these fragments (5,380 nucleotides) was about twice that expected (2,510 nucleotides) from electron-microscopic measurements of the DNA (ref. 5) (Table 1).

We also used a virus-specific double-stranded (ds) DNA, which we consider to be a replication intermediate, isolated from ³²P-labelled, BGMV-infected leaves. This contains the complementary strand to viral DNA and both strands are of

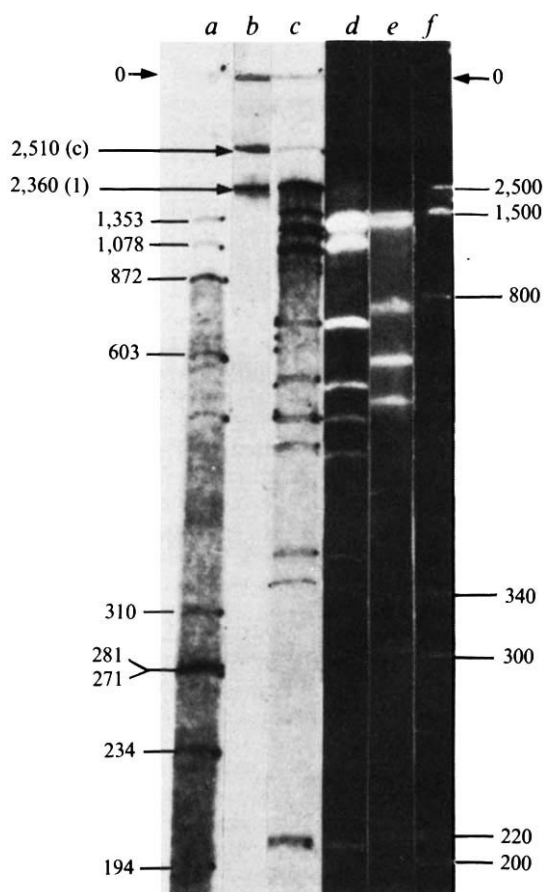


Fig. 1 Analysis of BGMV DNA by restriction endonucleases. *a*, 6 μ g of Φ X174 viral ssDNA was incubated with 70 units of *HaeIII* for 16 h at 37 °C in 50 mM Tris-HCl, pH 7.5 (120 μ l), 5 mM MgCl₂ and 0.5 mM dithiothreitol. *b*, 4 μ g of BGMV viral DNA was incubated as in *a*, but without restriction enzyme; *c*, circular DNA; *l*, linear DNA. *c*, 4 μ g of BGMV viral DNA was incubated with 120 units of *HhaI* for 16 h at 37 °C in 50 mM Tris-HCl (pH 8.0, 500 μ l) and 5 mM MgCl₂. *d*, 5,000 c.p.m. of ³²P-BGMV dsDNA, with 5 μ g of Φ X174 RF DNA as carrier, was incubated with 20 units of *HhaI* as in *c*. *e*, 5,000 c.p.m. of ³²P-BGMV dsDNA with 5 μ g of Φ X174 RF DNA as carrier was incubated with 20 units of *HaeIII* as in *b*. *f*, 2,000 c.p.m. of ³²P-M13 RF DNA, prepared by the procedure of Messing (unpublished protocol), with 5 μ g of Φ X174 RF DNA as carrier, was incubated as in *e*. After incubation, samples were phenol/chloroform extracted and the DNA ethanol-precipitated¹⁵, then redissolved in 10 μ l of sterile distilled water. Immediately before application to 4% polyacrylamide (acrylamide/bisacrylamide, 22:1) 7 M urea slab gels (0.75 mm $\times$ 13 cm $\times$ 14 cm)¹⁵, each sample was added to 6 μ l of a solution containing 50% sucrose, 30% urea, 0.2% xylene cyanol, 0.2% bromophenol blue and heated at 90 °C for 1 min. The electrophoresis buffer was 50 mM Tris-borate (pH 8.3), 1 mM EDTA. Electrophoresis was at 200 V until the xylene cyanol dye reached the bottom of the gel. Gels *a*, *b* and *c* were stained in the dark for 30 min with 0.005% Stains-All in 50% formamide, then destained in distilled water under light. For autoradiography, X-ray film (DuPont Cronex) was exposed to dried gels (*d*, *e* and *f*) in intensifying screen cassettes at -80 °C for $\sim$ 4 d before developing. Arrows indicate the positions of restriction fragments from *HaeIII* digests of Φ X174 viral DNA (left) and *HhaI* digests of M13 RF DNA (right); the numbers indicate approximate number of nucleotides (Φ X174) or nucleotide pairs (M13) of the fragments. Interpolated values for BGMV viral and dsDNA restriction fragments are given in Table 1. The top of the gel is indicated by the arrow labelled 0.

Table 1 Restriction fragment sizes of Φ X174 ssDNA, BGMV ssDNA, BGMV dsDNA and M13 RF DNA shown in Fig. 1, in order of increasing electrophoretic mobility

DNA	Treatment	Fragment sizes (nucleotides or nucleotide pairs)	Remarks
Φ X174 ss	<i>Hae</i> III	1,353; 1,078; 872; 603; 310; 281; 271; 234; 194*	Sizes of these fragments were computed from the nucleotide sequence given in ref. 18
BGMV ss	Untreated	2,510 (circular); 2,360 (linear)	Sizes based on electron microscope measurements ⁵
BGMV ss	<i>Hha</i> I	(2,510)†; (2,360); (1,650); 1,230; 1,050; (950); 660; 570; 520; 475; 365; 335; 175	Sizes based on interpolation using Φ X174 ssDNA <i>Hae</i> III fragments as standards. Sum of length of fragments in complete digests is 5,380 nucleotides
BGMV ds	<i>Hha</i> I	1,350; 1,030; 650; 520; 460; 420; 340; 320	Sizes based on M13 RF DNA <i>Hae</i> III fragments as standards. Sum of fragment lengths is 5,280 nucleotide pairs
BGMV ds	<i>Hae</i> III	1,480; 1,380; 750; 585; 510; 290	Sizes based on M13 RF DNA <i>Hae</i> III fragments as standards. Sum of fragment lengths is 4,995 nucleotide pairs
M13 ds	<i>Hae</i> III	2,500; 1,500; 800; 340; 300; 220; 200	Sizes of these fragments are from values given in a laboratory protocol (J. Messing, unpublished)

* Fragments 118 and 72 nucleotides long run off the gel in conditions used in our experiments (see Fig. 1 legend). Identity of these fragments was verified by comparison with *Hae*III restriction products of Φ X174 RF DNA. Other fragments in Fig. 1a, some of which occur as discrete bands, are due to the presence of ~20% by weight linear ssDNA in the Φ X174 ssDNA preparation.

† Values in parentheses are for DNA fragments produced by incomplete digestion of the substrate, as indicated by their absence in digests of BGMV dsDNA by the same enzyme, and by results of prolonged incubation of ssDNA with larger amounts of enzyme (not shown).

approximately unit length in relation to the circular viral DNA (M.I. and R.M.G., unpublished). The fragments produced by *Hha*I digestion of BGMV dsDNA corresponded exactly in number and size to those produced by complete *Hha*I digestion of circular viral ssDNA (Fig. 1).

Digestion of BGMV dsDNA with *Hae*III yielded six fragments with a total length of 4,995 nucleotide pairs (Table 1), confirming the results obtained with *Hha*I.

Attempts to analyse BGMV ssDNA by digestion with *Hae*III were not successful. *Hae*III digestion yields the same number and sizes of fragments from Φ X174 ssDNA as are found in *Hae*III digests of Φ X174 replicative form (RF) DNA¹⁶. The *Hae*III restriction fragments of circular BGMV viral DNA, however, were very small (20–100 nucleotides) and did not form discrete bands (data not shown). The nucleotide ratio of ³²P-labelled BGMV DNA is 32% A:33% T:22% G:13% C (unpublished results); thus it is unlikely that the *Hae*III recognition sequence (5'GG¹CC3') is as numerous in such a small DNA (with G+C=35%) as the restriction analysis suggested. Results with BGMV dsDNA confirm this. We cannot explain the apparently anomalous behaviour of *Hae*III with BGMV ssDNA.

These results indicated that BGMV may contain a divided genome with ssDNA components of the same physical size but different nucleotide sequence. The same result could be expected, however, if two related but distinct strains of the virus were present. We tested the divided genome hypothesis by investigating the dilution kinetics of infection by BGMV DNA in bean cell protoplasts. Protoplasts were prepared from leaf mesophyll cells of healthy beans (N.B.B. and R.M.G., unpublished results), inoculated with different amounts of BGMV DNA, and infected cells scored after 24 h by immunofluorescence. The slope of the infectivity dilution plot (Fig. 2) was closer to two than one.

Part of the initial evidence that led to the discovery of divided genomes in other plant virus groups were infectivity dilution curves whose slopes deviated significantly from unity^{19,20}. Our results now indicate that BGMV and possibly other geminiviruses may also contain a divided genome. This result extends to ssDNA viruses the interesting prevalence of divided genomes among the plant viruses and we believe it is the first example of a divided genome in a DNA virus. We conclude that the BGMV DNA genome is encapsidated in paired particles of two classes which, according to all present evidence, differ only in the sequence of the circular DNA molecule they contain. If this hypothesis is correct, the infectivity dilution kinetics of the virus particles should also approach a value of two; unfortunately, we have not found an experimentally useful local

lesion host for BGMV and attempts to infect protoplasts by inoculation with whole virus have been unsuccessful.

This discovery is expected to complicate further studies on the molecular biology of BGMV. To sequence or map gene functions on BGMV DNA, we must be able to resolve two DNA

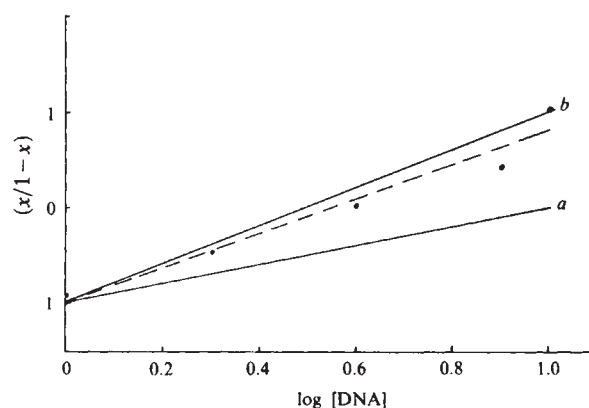


Fig. 2 Relationship between concentration of BGMV DNA in the inoculum and proportion of inoculated bean cell protoplasts infected. Plots shown are data from one (expt. 3) of five separate experiments (---) and theoretical plots (—) for *a*, one-hit and *b*, two-hit kinetics. Protoplasts were isolated from the mesophyll of Top Crop bean leaves and inoculated with various amounts of DNA (data are in $\mu\text{g ml}^{-1}$). Infected cells were scored by an indirect fluorescent-antibody method using anti-BGMV rabbit serum and fluorescein-labelled anti-rabbit γ -globulin goat serum. Data were corrected for the number of living cells determined by phenosafranin staining²¹ and then transformed to logit values²² ($\log x/(1-x)$) where *x* is the proportion of living cells infected. The logit transformation was used to correct for the decreased availability of infectable cells at higher DNA concentrations. Data from five experiments were plotted and the lines drawn by linear regression analysis. The slopes, correlation coefficients (*r*) and the probability level (*P*) at which *r* values are significant are shown below:

Expt	1	2	3	4	5
Slope	1.99	1.71	1.71	1.78	1.78
<i>r</i>	0.97	0.96	0.98	0.99	0.97
<i>P</i>	0.01	0.05	0.01	0.01	0.01

species which are indistinguishable by size but differ in nucleotide sequence. Perhaps the best approach would be to find restriction endonucleases that will digest at one site in only one of the two sequences and then to clone that sequence out of a mixture of the two using recombinant DNA techniques.

We acknowledge the collaboration and advice of Dr K. S. Browning, and thank Drs C. L. Niblett and R. W. Blakesley for discussions and Dr P. D. Shaw for comments on an earlier draft. Research was supported by a grant (7800549) from the USDA Competitive Research Grants Office and from the Illinois Agricultural Experiment Station (project 68-366).

Received 15 August; accepted 13 November 1980.

1. Goodman, R. M. in *Handbook of Plant Viral Diseases* (ed. Kurstak, E.) (North Holland/Elsevier, Amsterdam, in the press).
2. Matthews, R. E. F. *Intervirology* **11**, 133-135 (1979).
3. Goodman, R. M. *Nature* **266**, 54-55 (1977).
4. Harrison, B. D. *et al.* *Nature* **270**, 760-762 (1977).
5. Reisman, D., Ricciardi, R. P. & Goodman, R. M. *Virology* **97**, 388-395 (1979).
6. Francki, R. I. B., Hatta, T., Boccardo, G. & Randles, J. W. *Virology* **101**, 233-241 (1980).
7. Ward, D. C. & Tattersall, P. *Replication of Mammalian Parvoviruses* (Cold Spring Harbor Laboratory, New York, 1978).
8. Denhardt, D. T., Dressler, D. & Ray, D. S. *The Single-Stranded DNA Phages* (Cold Spring Harbor Laboratory, New York, 1978).
9. Bock, K. R., Guthrie, E. J. & Woods, R. D. *Ann. appl. Biol.* **77**, 289-296 (1974).
10. Galvez, G. E. & Castano, M. *Turrialba* **26**, 205-207 (1976).
11. Goodman, R. M., Shock, T. L., Haber, S., Browning, K. S. & Bowers, G. R. Jr *Virology* **106**, 168-172 (1980).
12. Hatta, T. & Francki, R. I. B. *Virology* **92**, 428-435 (1979).
13. Goodman, R. M., Bird, J. & Thongmeearkom, P. *Phytopathology* **67**, 37-42 (1977).
14. Goodman, R. M. & Bird, J. *AAB/CMV Descriptions of Plant Viruses* No. 192 (Commonwealth Mycological Institute, Kew, 1978).
15. Browning, K. S. thesis, Univ. Illinois (1980).
16. Blakesley, R. W. & Wells, R. D. *Nature* **257**, 421-422 (1975).
17. Horiuchi, K. & Zinder, N. D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 2555-2558 (1975).
18. Sanger, F. *et al.* *J. molec. Biol.* **125**, 224-246 (1978).
19. Fulton, R. W. *Virology* **18**, 466-485 (1962).
20. Van Vloten-Doting, L., Kruseman, J. & Jaspars, E. M. *Virology* **34**, 728-737 (1968).
21. Widholm, J. *Stain Technol.* **47**, 189-194 (1972).
22. Van der Plank, J. E. *Plant Disease: Epidemics and Control* (Academic, New York, 1963).

Plasmid R1 DNA replication dependent on protein synthesis in cell-free extracts of *E. coli*

Ramon Diaz & Kurt Nordström

Department of Molecular Biology, Odense University, DK-5230 Odense M, Denmark

Walter L. Staudenbauer

Max-Planck-Institut für Molekulare Genetik, Abt. Schuster, D-1000 Berlin 33, FRG

Bacterial plasmids serve as model systems for studying the regulation of DNA replication¹. Elucidation of the molecular mechanisms involved in plasmid DNA synthesis requires the development of efficient cell-free plasmid replication systems. Such *in vitro* systems have previously only been described for Col E1-type plasmids^{2,3} and for the R6K plasmid^{4,5}. Here we report that extracts of *Escherichia coli* can carry out the complete replication of miniplasmids derived from the antibiotic-resistance plasmid R1. This R1 replication system differs from the previously described ColE1 and R6K systems in its strict dependence on DNA-directed protein synthesis. We believe this to be the first report of the functional coupling of the three fundamental reactions of genetic information transfer (transcription, translation and replication) in a cell-free system.

Table 1 Requirements of the system

Omissions	dCMP incorporated (pmol)
None	94.9
MgCl ₂	0.8
dTTP	5.1
ATP	2.1
CTP, GTP, UTP	3.5
Creatine phosphate	1.0
Cyclic AMP	36.7
Amino acids	40.3

Extract was prepared from C600 (pKN177) and supplemented with exogenous pKN177 DNA (30 µg ml⁻¹). The complete system is the standard reaction mixture described in Fig. 1 legend with an additional 0.5 mM of each of the 20 amino acids. [α -³²P]dCTP (1,000 c.p.m. pmol⁻¹) was used as radioactive label. Reaction mixtures (25 µl) were incubated in duplicate at 30 °C for 60 min.

To develop an *in vitro* replication system for the R1 plasmid, we used the mini-R1 plasmids pKN177 and pKN182, which had been spontaneously generated from the R1 Cop mutant pKN104 (ref. 6). Plasmid pKN182 has a molecular weight of 3.9×10^6 and carries only the replication region of the parent plasmid, whereas pKN177, having a molecular weight of $\sim 9 \times 10^6$, also contains the ampicillin-resistance gene of R1. The Cop phenotype is caused by a single base substitution in the replication region⁷, which prevents the expression of a *trans*-acting

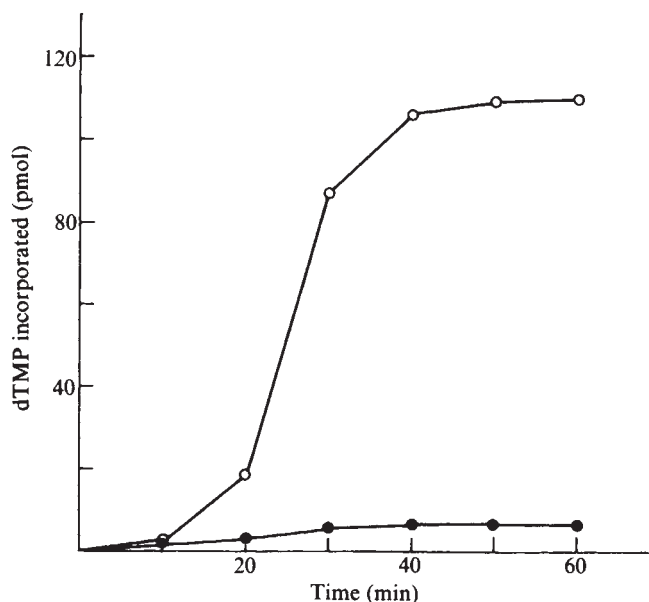


Fig. 1 Kinetics of pKN177 DNA synthesis. A cell-free extract was prepared from *E. coli* C600 (pKN177) by the freeze-thaw method⁸ in the presence of 1 mM EDTA and 1 mM dithiothreitol. Standard reaction mixtures contained final concentrations of 50 mM HEPES pH 8.0, 100 mM KCl, 11 mM magnesium acetate, 15 mM creatine phosphate, 0.1 mg ml⁻¹ creatine kinase, 2 mM ATP, 0.4 mM each of CTP, GTP and UTP, 0.05 mM NAD, 0.05 mM cyclic AMP and 0.025 mM each of dATP, dCTP, dGTP and ³H-dTTP (500 c.p.m. pmol⁻¹). Reaction mixtures (500 µl) containing 250 µl extract (7.5 mg protein) were incubated at 30 °C without (●) or with (○) addition of exogenous pKN177 DNA (30 µg ml⁻¹). At the times indicated, 25-µl samples were removed and assayed for acid-insoluble radioactivity.

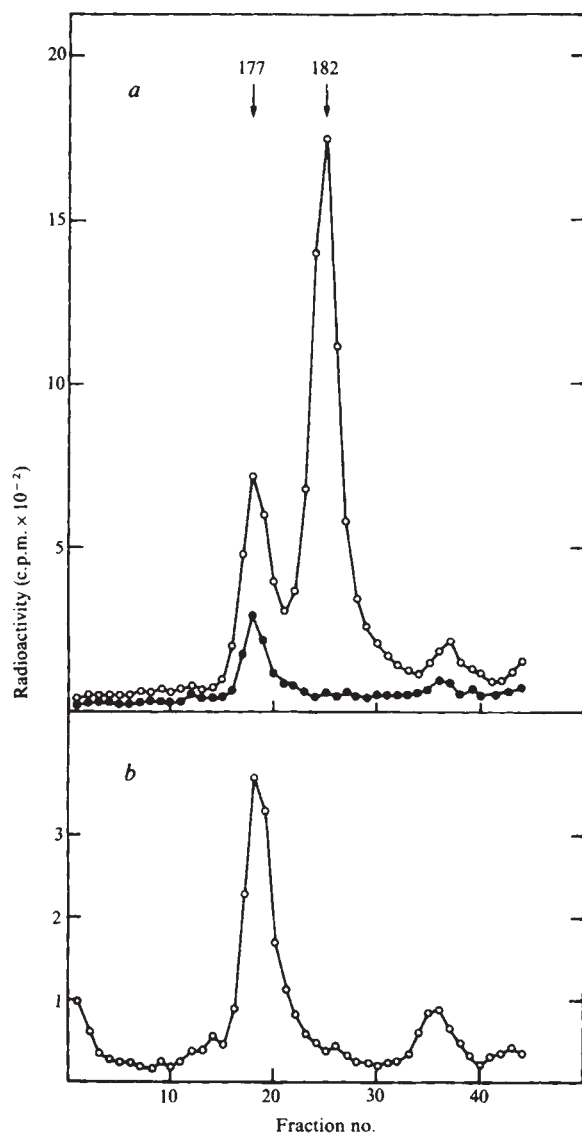


Fig. 2 Alkaline CsCl gradient centrifugation of *in vitro* synthesized DNA. Extracts were incubated for 60 min at 30 °C in standard reaction mixtures (150 μ l) without or with addition of exogenous plasmid DNA (30 μ g ml⁻¹). Reactions were stopped by addition of EDTA (50 mM) and the DNA was purified by gel filtration through a Sepharose 6B column. Aliquots were layered on preformed 4.2-ml alkaline CsCl gradients (density 1.2–1.4 g ml⁻¹ in 0.2 M NaOH) and centrifuged for 35 min in a Beckman SW 50.1 rotor at 5 °C. Fractions (100 μ l) were collected from the bottom of the tubes and assayed for radioactivity. Centrifugation is from right to left. The arrows indicate the positions of supercoiled pKN177 and pKN182 DNA, respectively. *a*, C600 (pKN177) extract incubated without (●) or with (○) addition of pKN182 DNA; *b*, C600 extract incubated with addition of pKN177 DNA.

negative control function⁶. Due to their reduced size and increased copy number (~25 copies per chromosome), these mini-R1 plasmids offer experimental advantages for setting up an *in vitro* system for R1 DNA replication.

Plasmid DNA synthesis was first studied in extracts from the plasmid-carrying *E. coli* strain C600 (pKN177) with the assumption that mini-R1 replication may require a plasmid-coded replication protein. We found that extracts prepared by freeze-thaw lysis of lysozyme-treated cells can catalyse a low but significant amount of dTMP incorporation into endogenous

Table 2 Replication capacity of extracts prepared from plasmid-carrying and plasmid-free strains

Source of extract	Plasmid DNA added		
	None	pKN177	ColE1
C600 (pKN177)	14.8	138.1	182.9
C600	0.0	9.5	163.5

Standard reaction mixtures (25 μ l) included ³H-dTTP (500 c.p.m. pmol⁻¹) and 0.5 mM of each of the 20 amino acids. Exogenous pKN177 DNA (30 μ g ml⁻¹) or ColE1 DNA (50 μ g ml⁻¹) were added as indicated. Incubations were performed in duplicate at 30 °C for 60 min. Values shown are dTMP incorporated (pmol).

plasmid DNA. Furthermore, a marked stimulation of DNA synthesis was observed on addition of exogenous pKN177 DNA. The kinetics of dTMP incorporation with or without addition of plasmid DNA is shown in Fig. 1. It can be seen that there is a lag period of 10 min before the onset of DNA synthesis and that the incorporation levels off after 40 min of incubation. Addition of pKN177 DNA stimulates the total incorporation

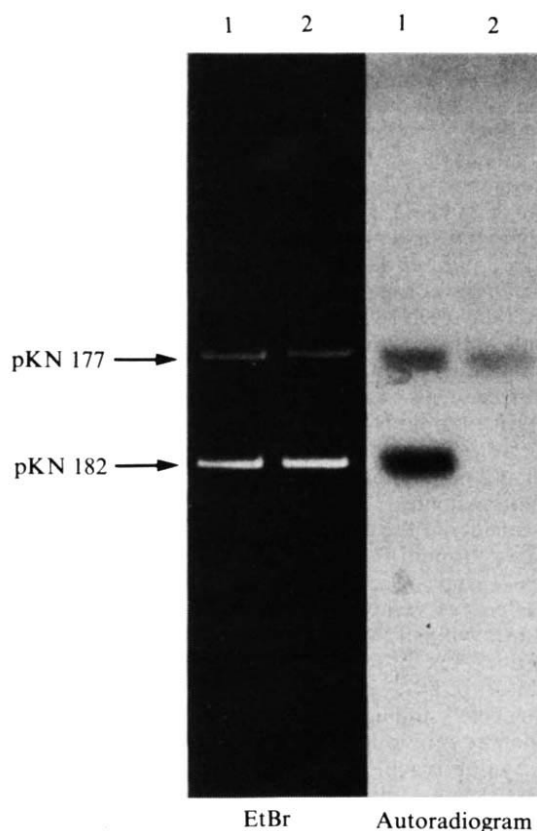


Fig. 3 Effect of rifampicin on plasmid DNA replication. Extract from C600 (pKN177) was incubated for 60 min at 30 °C in standard reaction mixtures (150 μ l) containing [α -³²P]-dCTP (1,000 c.p.m. pmol⁻¹) plus exogenous pKN182 DNA (30 μ g ml⁻¹) without or with addition of rifampicin (25 μ g ml⁻¹). The reactions were stopped with EDTA and the DNA was purified as described in Fig. 2 legend. Aliquots of the untreated control (lane 1) and of the rifampicin-treated sample (lane 2) were electrophoresed in a 1.0% agarose gel for 3.5 h at 100 V. The gel was stained with ethidium bromide and autoradiographed. The arrows indicate the positions of supercoiled pKN177 and pKN182 DNA, respectively.

~15-fold. The optimal concentration of exogenous plasmid DNA is about 3 nM; higher concentrations of pKN177 DNA are inhibitory. Similar results were obtained with pKN182 DNA (data not shown).

The requirements of the system (supplemented with exogenous pKN177 DNA) are summarized in Table 1. It was found that the magnesium ion concentration is of critical importance. Optimal dTMP incorporation requires 9–11 mM MgCl₂, whereas almost no DNA synthesis can be detected at 5 or 15 mM MgCl₂. In addition, the system requires not only deoxynucleoside triphosphates but also all four ribonucleoside triphosphates and is strictly dependent on an ATP-regenerating system. A variable degree of stimulation, depending on the extract preparation, was noted on adding cyclic AMP and/or an amino acid mixture. The replicative capacity for mini-R1 DNA is lost if the extract is prepared in the absence of dithiothreitol.

Having optimized the reaction conditions, we investigated whether exogenous pKN177 DNA can also replicate in extracts from *E. coli* cells that do not carry any plasmid. It was found that extracts from plasmid-free cells can catalyse a significant amount of DNA synthesis when supplemented with pKN177 DNA (Table 2). However, the replicative capacity for pKN177 DNA of such extracts is an order of magnitude lower than that of extracts from cells carrying this plasmid. In comparison, exogenous ColE1 DNA is replicated by both types of extracts with similar efficiency.

To characterize the reaction product, the *in vitro* synthesized DNA was analysed by alkaline CsCl gradient centrifugation (Fig. 2). To distinguish between endogenous and exogenous DNA, extracts from C600 (pKN177) were supplemented with pKN182 DNA. Comparison of the two superimposed gradients shown in Fig. 2a indicates that the exogenously added pKN182 DNA not only functions as template for its own replication but also stimulates the synthesis of the endogenous pKN177 DNA. This stimulation is R1 specific as it was consistently observed on addition of mini-R1 plasmids but not on addition of ColE1 DNA (data not shown).

The incorporated label is found in two peaks of fast-sedimenting DNA, corresponding to closed-circular monomers of pKN177 and pKN182. Only a minor fraction of the label sediments in the position of open-circular DNA. Similarly, the reaction product of pKN177 DNA replication in extracts from plasmid-free cells consists predominantly of supercoiled monomeric plasmid DNA (Fig. 2b). No peak of labelled DNA fragments sediments more slowly than unit-length single strands, as would be expected for material derived from replicative intermediates^{4,8}. These results indicate that the *in vitro* system carries out the complete replication of both endogenous and exogenous mini-R1 DNA without detectable accumulation of partially replicated molecules.

The effects of various antibiotics on mini-R1 replication *in vitro* are shown in Table 3. As expected for semi-conservative DNA replication, the incorporation of dTMP is entirely blocked by inhibitors of DNA gyrase (novobiocin and oxolinic acid)^{9,10}. An unexpected finding is the complete inhibition of mini-R1 replication by antibiotics which interfere with different stages of protein synthesis (chloramphenicol, puromycin, streptomycin and tetracycline). Plasmid replication evidently is coupled tightly to translation, because DNA synthesis stops within 10 min of the addition of chloramphenicol (data not shown). Plasmid DNA synthesis is also strongly affected by rifampicin, a specific inhibitor of RNA polymerase. The residual rifampicin-resistant incorporation observed with extracts from plasmid-carrying (but not plasmid-free) cells is probably due to the presence of endogenous plasmid-specific RNA.

The inhibitory effect of rifampicin on plasmid replication in an extract containing endogenous pKN177 DNA and exogenous pKN182 DNA was further analysed by agarose gel electrophoresis of the reaction products (Fig. 3). Comparison of the ethidium bromide-stained gel with the autoradiogram shows that the ³²P-label is incorporated exclusively into supercoiled plasmid DNA. Interestingly, only the endogenous pKN177

Table 3 Effects of antibiotics on pKN177 DNA synthesis

Addition ($\mu\text{g ml}^{-1}$)	Source of extract	
	C600 (pKN177)	C600
None	101.7	13.8
Novobiocin (25)	1.6	0.2
Oxolinic acid (50)	2.4	0.3
Chloramphenicol (100)	1.1	0.3
Puromycin (100)	1.5	0.2
Streptomycin (100)	1.6	—
Tetracycline (100)	1.4	—
Rifampicin (25)	14.9	0.1

Standard reaction mixtures (25 μl) were supplemented with exogenous pKN177 DNA (30 $\mu\text{g ml}^{-1}$) and 0.5 mM of each of the 20 amino acids. ³H-dTTP (500 c.p.m. pmol^{-1}) was used as radioactive label. Incubations were performed in duplicate at 30 °C for 60 min. Values shown are dTMP incorporated (pmol).

DNA but not the exogenous pKN182 DNA can function as a template for rifampicin-resistant DNA replication.

The data presented here demonstrate that mini-R1 replication *in vitro* requires both transcription and translation. A requirement for *de novo* RNA and protein synthesis has previously been observed *in vivo* for the uncontrolled replication of a runaway-replication mutant of R1 (ref. 11). It was postulated that the translation product is an R1-specific initiator protein that is not re-usable. Indirect evidence that R1 carries a gene essential for autonomous replication has been presented by Kollek *et al.*¹². This gene is considered analogous to the *rep A* gene of the closely related R100 plasmid¹³. Evidently, mini-R1 replication *in vitro* is also strictly dependent on the plasmid DNA-directed synthesis of an essential replication protein. The R1-specific stimulation of endogenous plasmid replication by addition of exogenous mini-R1 DNA can thus be explained by assuming that the presumptive *rep A* gene product is a *trans*-acting protein. The transcription requirement would then reflect the synthesis of a messenger RNA for this protein. On the other hand, the differential effect of rifampicin on endogenous and exogenous plasmid DNA synthesis (see Fig. 3) indicates that a *cis*-acting RNA associated with the template DNA may also be involved in R1 DNA replication.

Results similar to those presented here for pKN177 and pKN182 have also been obtained for other mini-R1 plasmids (R.D., unpublished data). The plasmids tested include pKN1562 the copy control of which corresponds to the parent R1 plasmid¹⁴. Therefore, this *in vitro* system is not restricted to a particular type of Cop mutant and should prove useful for a detailed analysis of R1 replication control.

We thank Dr Soren Molin for supplying the plasmids pKN177 and pKN182 and for helpful suggestions, and Dr Heinz Schuster for providing support and laboratory facilities. R.D. was supported by a short-term fellowship from EMBO and a grant from the Danish MRC.

Received 15 August; accepted 21 November 1980.

- Kolter, R. & Helinski, D. R. *A. Rev. Genet.* **13**, 355–391 (1979).
- Tomizawa, J. in *DNA Synthesis: Present and Future* (eds Molineux, I. & Kohiyama, M.) 797–826 (Plenum, New York, 1978).
- Staudenbauer, W. L. *Curr. Topics Microbiol. Immun.* **83**, 93–156 (1978).
- Inuzuka, M. & Helinski, D. R. *Biochemistry* **17**, 2567–2573 (1978).
- Inuzuka, M. & Helinski, D. R. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5381–5385 (1978).
- Molin, S. & Nordström, K. *J. Bact.* **141**, 111–120 (1980).
- Stougaard, P., Molin, S., Nordström, K. & Hansen, F. G. *Molec. gen. Genet.* (in the press).
- Staudenbauer, W. L. *Molec. gen. Genet.* **145**, 273–280 (1976).
- Staudenbauer, W. L. *J. molec. Biol.* **96**, 201–205 (1975).
- Staudenbauer, W. L. *Eur. J. Biochem.* **62**, 491–497 (1976).
- Uhlén, B. E. & Nordström, K. *Molec. gen. Genet.* **165**, 167–179 (1978).
- Kollek, R., Oertel, W. & Goebel, W. *Molec. gen. Genet.* **162**, 51–57 (1978).
- Yoshikawa, M. *J. Bact.* **118**, 1123–1131 (1974).
- Molin, S., Stougaard, P., Uhlén, B. E., Gustafsson, P. & Nordström, K. *J. Bact.* **138**, 70–79 (1979).

Highly conserved lipoprotein assembly in teleost and amphibian yolk-platelet crystals

R. H. Lange

Institute for Anatomy and Cytobiology, University of Giessen, Aulweg 123, D-6300 Giessen, FRG

Ohlendorf *et al.*¹ have reported that yolk-platelet crystals in the frog, *Xenopus laevis*, were orthorhombic (space group $P2_12_12_1$) and consisted of elongated asymmetric units ($\sim 5.5 \times 11.5 \times 25$ nm). The presence of an orthorhombic lattice in amphibian yolk platelets fulfilling the symmetry requirements of crystals had been missed in earlier structural interpretations^{2,3}. A comparative study of teleost, urodele and anuran yolk-platelet crystals performed in our laboratory has shown the widespread occurrence among aquatic vertebrates of an orthorhombic lattice with unit-cell sides similar to those of *Xenopus*. However, we report here that the space group ($P2_12_12_1$) and certain features of the macromolecular complex are at variance with those reported for *Xenopus*¹.

The material studied consisted of the amphibians *Rana temporaria* (gastrula) and *Triturus* sp.⁴ (uncleaved, two-cell and blastula stages) and the teleost *Pelvicachromis pulcher*⁵ (Cichlidae; immature and mature eggs). Thin-sectioned specimens were fixed (1% glutaraldehyde/1% OsO_4 /0.1 M phosphate buffer) and subjected to two very different embedding procedures (epoxy-resin and glutaraldehyde-urea⁶). On applying electron diffraction and tilting, the presence of an orthorhombic lattice was confirmed in all three cases. This was achieved by establishing the presence of symmetry mmm for the reciprocal orthorhombic lattice in each case. Moreover, the unit-cell sides a , b , c were almost identical among the three species studied and—falling in the range derived by Ohlendorf *et al.*^{1,7} for wet and lyophilized platelets—were also very close to those of *X. laevis* (Fig. 1 legend). This observation supports our view that we are dealing with much the same structure in teleost and amphibian yolk platelets and that our data are also relevant to

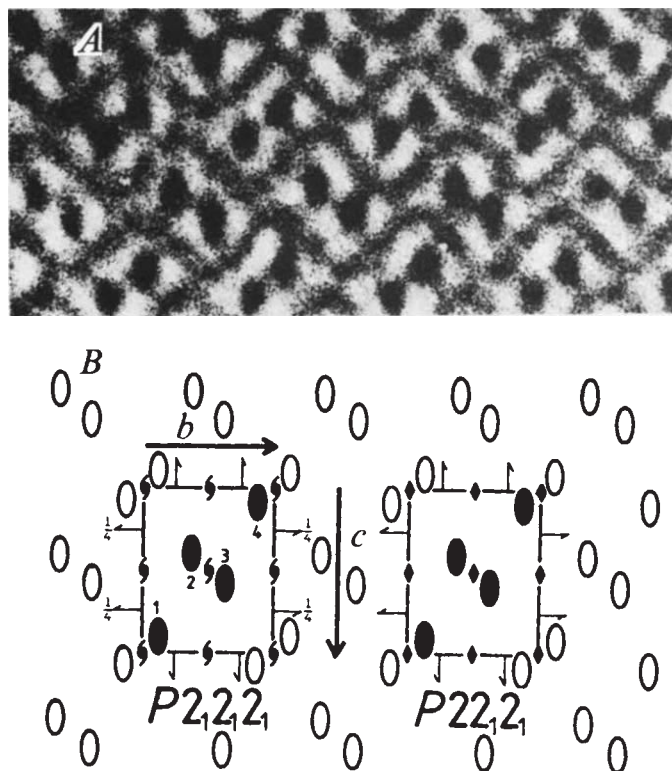


Fig. 2 Electron micrograph of a teleost yolk-platelet crystal viewed along the short axis a of the unit cell (A) and tracings of the same [100] projection (B) made approximately to the same scale. Orientation and size of the unit cell have been unequivocally defined by corresponding diffraction patterns; the cell may, however, be displaced. The footprint-like pattern shown can only be interpreted by assuming 2₁-screw-axes parallel to the unit-cell sides b and c . Note that these two screw-axes have unique positions in this projection, there are no alternative positions for them. If the primitive orthorhombic lattice has been correctly assigned then only two space group interpretations are possible¹¹ ($P2_12_12_1$ and $P22_12_1$, but not $P2_12_12_1$); these interpretations are illustrated in B. $P22_12_1$ implies a 2-fold symmetry axis ($\bullet$) perpendicular to the plane of the paper in the middle of each pair of dark elliptic bodies and thus a location of these bodies in the same height along this axis; $P2_12_12_1$ has a 2-fold screw-axis ($\bullet$) at this site, implying that the height of neighbouring dense bodies differs by $a/2$, which is in fact shown to be the case (Fig. 3). A is a high-power enlargement of a raw electron micrograph originally recorded on 35-mm film at magnification $\times 25,000$ and shows a certain degree of distortion due to sectioning. Dense particles (probably phospholipid complexes, see text) are numbered in the same way as in Fig. 3B. International symbols¹¹; $b \sim 16.5$ nm.

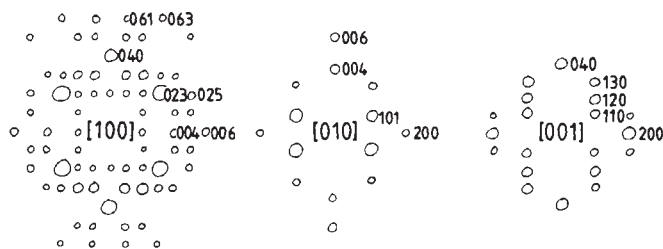


Fig. 1 Electron diffraction patterns (tracings) obtained in *Triturus* sp. when the crystals were oriented as indicated. In the other two species studied, only changes in the intensities of single reflections were noted. The means of unit-cell lengths as determined from such patterns in the three species had the following ranges (in parentheses the X-ray data from *X. laevis* for dried⁷ and wet¹ preparations are given for comparison): $a = 8.3 \dots 9.1$ (7.9 ... 8.9) nm; $b = 15.8 \dots 17.7$ (15.8 ... 17.2) nm; $c = 16.6 \dots 19.3$ (17.6 ... 19.6) nm; the lower limit of our data refers to epoxy-resin embedding following dehydration in acetone, the upper limit to glutaraldehyde-urea embedding in the partly hydrated state.

the findings in *Xenopus*^{1,7}. The evolutionarily conservative behaviour of the molecular assembly is underlined by the very similar appearance of 12 indexed diffraction patterns (for [100], [010], [001], see Fig. 1) as well as seven identified crystal projections (electron images) in the three species studied.

The absence of reflections (100, 300, 010, 030, 050, 001, 003, 005, 007) in the electron diffraction patterns suggests the presence of 2-fold screw-axes parallel to all three unit-cell sides, that is, space group $P2_12_12_1$. Conclusive proof for the space group, however, can be obtained in this case from the mass distribution in electron micrographs (Figs 2, 3). The most revealing projection with respect to molecular arrangement is the previously unstudied projection along the a axis ([100], Fig. 2), the second most revealing that along the c axis ([001], Fig. 3). It is evident from Figs 2 and 3 that there are four asymmetric

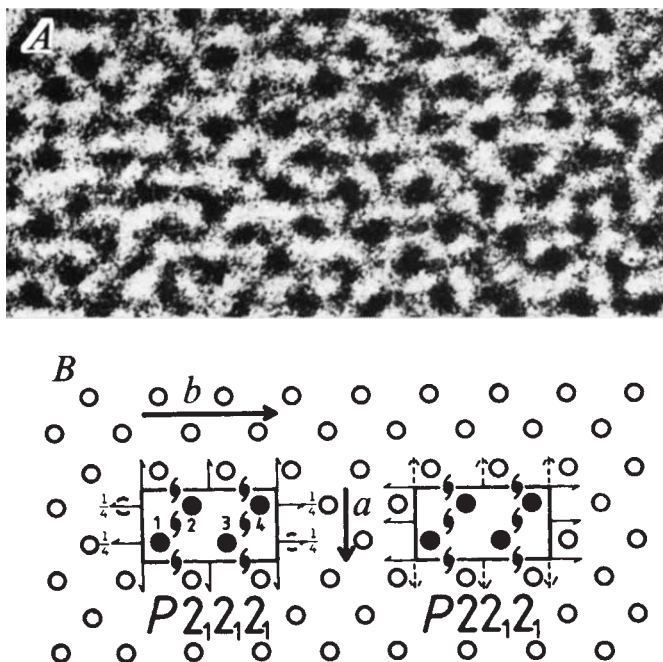


Fig. 3 Electron micrograph of a newt platelet crystal viewed along the c axis (A) and schematic tracing of the same [001] projection (B) made approximately to the same scale. It can be seen that only a screw diad (space group $P2_12_12_1$) and not a rotation diad (space group $P22_12_1$) parallel to unit-cell side a correctly describes the matter distribution. Note that positioning of these additional symmetry elements is not deliberate for geometrical reasons¹¹. The rotation diad not consistent with the observed mass distribution is shown dashed on the right half of B. Compare A with the negatively stained preparation in Fig. 1 of the report by Ohlendorf *et al.*¹. For further details see Fig. 2 legend.

units per unit cell, as in *X. laevis*¹. There is no evidence that the complex exceeds the length of the b axis¹; however, this point awaits confirmation by further analysis, as does the positioning of a local 2-fold axis¹ of the dimeric structure⁸.

To explain the discrepancies between the findings in *X. laevis*^{1,7} and our amphibian and teleost data, we should consider the methods used. As shown by investigations using negative staining techniques in electron microscopy of protein molecules⁹, there is commonly an overlap of negative and positive staining of such preparations. Such overlap is inevitable and must be considerable when a highly anionic protein like the phosvitin moiety⁸ of the yolk-platelet subunit is stained with a cationic dye containing uranyl ions¹. The dark spots in the positively stained crystals in [100] and [001] projection (Figs 2, 3)—unrecognizable in unstained sections but quite evident in sections stained with uranyl acetate at low pH (3.5)—give the most probable location of the phosvitin moiety²; in contrast to previous views², however, it accommodates both phosvitin molecules of the dimeric complex⁸. This significant part of the complex may largely have escaped representation in the negatively stained preparations of *X. laevis* platelets¹. In fact, the negatively stained preparation (Fig. 1 of ref. 1) is almost identical to the [001] projection (Fig. 3) in the three species studied here following positive staining of crystal sections with uranyl acetate and lead citrate. In a negatively stained preparation, the space occupied by stain is interpreted as being void of the macromolecule under study; this makes it very unlikely that the results of the three-dimensional reconstruction of the amphibian lipovitellin-phosvitin complex¹ is reliable.

The procedure used in the present study yields results comparable with X-ray data of protein crystals¹⁰, except that it

produces a reduction in length of some 10% (due partly to dehydration, especially for epoxy-resin embedding), various degrees of distortion (due to sectioning) and a resolution limit between 1.5 and 2 nm. All our findings were reproduced—and most of our work was indeed done—using glutaraldehyde-urea embedding in the partially hydrated state⁶. The original authors of this procedure have proved that the extremely lipid-rich myelin sheath does not lose its natural birefringence and dimensions when subjected to this embedding method⁶. This implies that the material studied in our laboratory has been preserved as adequately as possible. The sectioning process, furthermore, precludes the bias produced by splitting isolated platelets along preferential planes¹ and thus makes all crystal projections available with the same probability.

I conclude that teleost and amphibian yolk platelets are quite similar assemblies of lipoprotein complexes (orthorhombic, $P2_12_12_1$, almost identical unit-cell lengths for all species studied so far) and, therefore, contain a macromolecular constituent of highly conserved tertiary structure. Space group $P2_12_12_1$, and the three-dimensional shape of the asymmetric unit as described in isolated yolk platelets of *X. laevis*¹ do not agree with observations made on our comparative material.

I thank Professor S. Haussühl for crystallographic advice, Mr G. Magdowski for technical assistance and the Deutsche Forschungsgemeinschaft for support.

Received 1 July; accepted 20 November 1980.

1. Ohlendorf, D. H., Wrenn, R. F. & Banaszak, L. J. *Nature* **272**, 28–32 (1978).
2. Wallace, R. A. *Biochim. biophys. Acta* **74**, 505–518 (1963).
3. Honjin, R. in *Recent Progress in Electron Microscopy of Cells and Tissues* (eds Yamada, E. *et al.*) 95–108 (Thieme, Stuttgart & Igaku Shoin, Tokyo, 1976).
4. Lange, R. H. *Proc. 7th Eur. Congr. Electron Microscopy*, Vol. 2 (eds Brederoo, P. & de Priester, W.) 624–625 (Leiden, 1980).
5. Lange, R. H. *Cell Tissue Res.* **209**, 511–513 (1980).
6. Peterson, R. G. & Pease, D. C. *J. ultrastruct. Res.* **41**, 115–132 (1972).
7. Ohlendorf, D. H., Collins, M. L. & Banaszak, L. J. *J. molec. Biol.* **99**, 143–151 (1975).
8. Taborsky, G. *Adv. Protein Chem.* **28**, 1–210 (1974).
9. Unwin, P. N. T. *J. molec. Biol.* **98**, 235–242 (1975).
10. Lange, R. H., Blödorn, J., Magdowski, G. & Trampisch, H. J. *J. ultrastruct. Res.* **68**, 81–91 (1979).
11. *International Tables for X-Ray Crystallography* Vol. 1, 104–105 (Kynoch, Birmingham, 1969).

Corrigenda

In the letter 'West Antarctic ice sheet fluctuations in the Antarctic Peninsula area' by D. E. Sugden and C. M. Claperton, *Nature* **286**, 378–381 (1980), the correction factor of ~750 radiocarbon years for Antarctic Peninsula shells was added to rather than subtracted from the Holocene ¹⁴C dates of 6,930 ± 60 and 7,200 ± 50 yr BP for the outer and inner fractions of SRR-1500. The adjusted dates should be 6,180 and 6,450 radiocarbon years BP respectively. The discussion concerning the possibility of an ice-free George VI Sound in the Holocene thus refers to conditions 6,000–6,500 radiocarbon years ago and not ~8,000 years ago. This change does not affect the conclusions of the paper.

In the letter 'Selective killing of mycoplasmas from contaminated mammalian cells in cell cultures' by Marcus *et al.*, *Nature* **285**, 659–661 (1980), the footnote to Table 2 should refer to a 400-W lamp, not 40-W as published.

Erratum

In the letter 'Expression of H-2, lamin and SV40T and TASA on differentiation of transformed murine teratocarcinoma cells' by Knowles *et al.*, *Nature* **288**, 615–618 (1980), two lines were omitted from the bottom of the left-hand column on page 617; it should read:

..... differentiates seems to express the H-2D^b private specificity. It has been observed that somatic cell hybrids between F9 and differentiated cells can

BOOK REVIEWS

... and when the cupboard is bare?

Kenneth E. Boulding

THESE two books have a somewhat similar theme, but are about as different in style and approach as two books could be. The Krenz volume has a somewhat narrower scope, dealing primarily with energy, whereas that by Barnet takes materials into account; the basic theme of adaptation to the exhaustion of non-renewable resources is common to them both. The book by Krenz is sober, well documented, is based solidly on the best available quantitative information, and presents a rounded picture, even though the author is not afraid to express value judgments on such matters as the future of nuclear energy. The Barnet volume is sparkling, anecdotal in style, rarely presents a complete, well-rounded picture, and is constantly engaged in implied, not too explicit value judgments.

Krenz begins with a clear general consideration of the energy crisis and energy conservation, goes on to a thoughtful discussion of environmental and resource constraints, analyses the situation in regard to fossil fuels, then has a key chapter on more effective energy utilization, which is a better way of describing "conservation". There follows a discussion of "Nuclear Energy: A Faustian Bargain?" which expresses the anxieties and ambiguities involved without dismissing them. Then there is an excellent section on alternative sources of energy, which is both sober and sobering to the over-optimistic. The book concludes with a discussion of the need for a new ethic to deal with the probability of a more austere future.

The Barnet book starts with a rather uneven discussion of the "Post-Petroleum World", and continues with a section on "Guns, Butter and Oil: Changing the Face of Power", which also deals with minerals, food and water, and relates increasing scarcity to what he calls "the new international military order", particularly in the Third World. He concludes with a

The Lean Years: Politics in the Age of Scarcity. By R. Barnet. Pp.349. (Simon and Schuster: 1980.) \$12.95. To be published in the UK, in April, by Abacus, pbk £2.50. *Energy: From Opulence to Sufficiency.* By J. H. Krenz. Pp.264. (Hemisphere/Holt, Rinehart & Winston: 1980.) \$24.95, £15.50.

section entitled "The Global Factory: The Planning of Scarcity", in which he foresees a world employment crisis resulting from the disruption of traditional agriculture and the population explosion. This last chapter ends with a rather vague plea for decentralization without any clear indication as to how to achieve it. Here, Barnet sees little reason for optimism, and what emerges is the general feeling that bad boys will continue to be bad because whoever stops them will be worse. It is, indeed, a gloomy end to liberal hopes.

In terms of suggested solutions to the problem, Krenz, as befits the somewhat narrower scope of his book, lays great stress on the value of conservation and the large technical potential which is latent in conservation practices, in just getting more in terms of human values out of the same input of energy. There are estimates which suggest that we can maintain much the same style of life that we do now with 25 per cent of our current energy input, or with a bit of luck and technical change, maybe 50 per cent. This, in a sense, is a common task which must be shared widely. Barnet, on the other hand, leans towards conspiracy theories of what is the matter with us, does not lay so much stress on conservation, and ends up arguing for a rather vague kind of decentralization as a way of saving us from the concentrated power either of large corporations or of socialist states.

The contrast between the two books illustrates at least one of the virtues of quantified data. The Krenz book, even though it is somewhat abstracted from reality, does give us an overall picture of the proportions of the problem, which the qualitative anecdotal approach of Barnet does not really permit. There is a basic difference, perhaps, between the scientific and the journalistic approach.

Both books would be improved by a little more economics. Although neither of the authors is an economist, Krenz recognizes the importance of economic considerations, though he is perhaps a little over-impressed with purely physical concepts of efficiency. From the human point of view, the only significant concepts of efficiency are those which involve human values. Thus, a process can be highly inefficient in

terms of transformation into a particular form of energy, and even highly inefficient according to second law efficiency, that is, transformation of the availability of energy for work. But if the energy output is important by human standards and the input is low-valued, the process can be very efficient in human valuation terms. Thus, the rise of electric power, especially after about 1910, was inefficient by some physical standards but was extremely efficient in terms of human valuations. In commodity output at least, as measured by gross national product, per unit of energy input increased sharply in those decades, largely because of the greater convenience of subdivisibility of electricity as compared to alternative means of transporting energy. Both volumes neglect the problem of energy transport, which is most important from the point of view of human valuations. Energy when and where and in the form in which we want it almost invariably involves its transportation.

Energy: From Opulence to Sufficiency by Krenz can be strongly recommended to those who want an overall survey of the technological problems of energy in fairly non-technical language. The Barnet volume, *The Lean Years*, would appeal to those who enjoy liberal-radical rhetoric, who like to think that everything that is wrong with us is somebody else's fault or the result of a defective political structure of society, which no doubt it often is. Neither book, however, really comes to grips with the way in which decision making and the great network of social interaction actually move us towards overall betterment or worsening. But this, no doubt, is too much to ask.

Kenneth E. Boulding is Distinguished Professor of Economics Emeritus and Director of the Program of Research on General Social and Economic Dynamics at the Institute of Behavioral Science, University of Colorado, Boulder.

Jerrold Krenz — sober and thoughtful



Richard Barnet — sparkling and anecdotal



Progress and prospects in tumour virology

Peter W. J. Rigby

DNA Tumor Viruses. Molecular Biology of Tumor Viruses, Part 2, 2nd Edn. Edited by J. Tooze. Pp.945. (Cold Spring Harbor Laboratory: 1980.) \$65, \$78 outside USA.

THE first edition of this monograph on the molecular biology of tumour viruses was both timely and influential. It was timely because its publication, in 1973, coincided with the advent of techniques for the physical mapping of viral genomes and it was influential because it acted as an invaluable source for the many molecular biologists, including myself, who began working on tumour viruses in the hope that they would, in conjunction with the new techniques, provide insights into the mechanisms of eukaryotic gene regulation. This second edition thus had much to live up to and it has, in general, succeeded most admirably.

Like its predecessor, the book is a compilation of chapters written by a variety of authors and brought together under the editorship of John Tooze. In his preface Tooze admits to some of the problems that bedevilled the preparation of the book; in particular, it proved impossible to persuade so many authors to produce their material at exactly the same time. While many of these problems have clearly been resolved, there remain cases where it is apparent that closely related chapters were written, or finally revised, at quite different times and there are thus inconsistencies of fact or interpretation. These deficiencies are, however, more than compensated by the book's strengths. It aims successfully at a wide audience, being written in a clear, didactic style quite approachable by final-year undergraduates, yet it is also sufficiently comprehensive and penetrative to be of great value to established workers in the field.

The first section comprises six chapters concerned with the smallest DNA tumour viruses, the papovaviruses and papillomaviruses. Viruses of the former class are now understood in considerable detail which is reflected in separate chapters on genomic organization, the lytic cycle, cellular transformation and genetics. Each of these contributions was written by different authors which leads to some unevenness of approach due principally, one suspects, to the writing having been done at different times. This shows particularly in the treatment of the middle T-antigen of polyoma and of the cellular 53K protein found in SV40-transformed cells. I particularly enjoyed the chapter by Nick Acheson on the lytic cycle of SV40 and polyoma. This deals in some detail with experimental problems and gives the reader an excellent feel for the certainties and uncertainties of the area. The chapters on human papovaviruses and on papillomaviruses are quite different in content, being

necessarily much more biological in approach. However, the molecular biology of the human papovavirus BK is dealt with poorly; its DNA sequence, which appears in one of the appendices, is hardly discussed.

Adenoviruses are covered in three chapters, two written by Jane Flint alone, the other by her in collaboration with Tom Broker. That one author was involved in all three chapters shows clearly in a much more uniform approach and consistent treatment. The chapter on lytic infection is truly excellent, being both comprehensive and detailed yet clearly written and easy to follow. It also deals superbly with some of the less-known regions of tumour virus research, including an outstanding discussion of adenovirus assembly.

The chapter on herpes simplex viruses, by Patricia Spear and Bernard Roizman, is by far the largest in the book and is also the least satisfactory. It would have been much more appropriate for a specialized review. In many places the textual discussions would have benefited from the provision of appropriate diagrams while, conversely, it is the only chapter to use extensive figures of primary experimental data. These are discussed in a style so condensed that it will be impenetrable to all but experts. The chapter is also somewhat unbalanced. It deals too superficially with herpes genetics and is too heavily biased towards the work and opinions of the authors.

The final pages are occupied by six appendices giving the DNA sequences of SV40, polyoma and BK, and of the transforming segments of adenovirus types 5, 7 and 12. Also presented are lists of

restriction sites, maps of mRNAs and putative protein sequences. This section represents an excellent innovation and will be of the greatest value. I have only one criticism of this section, albeit a severe one. The sequence of SV40 has recently been modified by the addition of an extra, small restriction fragment. While this is referred to in the introduction to the appendix, the sequence used does not include it. It would have been worth correcting this, even at the price of a slight delay in publication.

This book is as timely as its predecessor, detailing the results of one phase of the work and setting the scene for the studies that are to come. It will, therefore, be as influential. I fully share the editor's regret that the increased size of the book has necessitated a significant increase in price and sincerely hope that a paperback edition will be forthcoming so as to put the book within the reach of the many students who ought to own a copy. Economics notwithstanding, this book is mandatory reading for those wishing to obtain a general view of tumour virology, for those wishing to become involved in this most exciting area of research and for those currently involved in it. The editor and the authors are to be congratulated on undertaking so well what must have seemed to them an almost impossible task. I can only hope, on behalf of all in the field, that in five years time they will have the courage to attempt a third edition. []

Peter W. J. Rigby is a member of the Cancer Research Campaign Eukaryotic Molecular Genetics Research Group and a Lecturer in Biochemistry at the Imperial College of Science and Technology, University of London.

Just beyond the pale

Paul Davies

Gravity, Particles, and Astrophysics. By Paul S. Wesson. Pp.188. (Reidel: 1980.) Dfl.65, \$34.

COSMOLOGY and astrophysics attract an enormous number of speculative theories, not all proposed by cranks, but many of them written somewhat tongue-in-cheek. Paul Wesson is a man with an encyclopaedic knowledge of those grey areas somewhere between the canonical models of orthodoxy, and the manifestly absurd, that manage to slip into the reputable journals. His previous book, *Cosmology and Geophysics* (Adam Hilger, 1978), dealt lucidly with a variety of "variable *G*" theories, in which the Newtonian gravitational *G* is assumed to vary with time. It is a theme continued and

updated in this latest work, but accompanied by a range of other, similar, conjectures: particle creation, anomalous redshifts, tired light and cosmic numerology, for example.

The author's subjects range ambitiously from the planets to the primaeval furnace. Scarcely an unorthodox idea escapes attention somewhere. The professional cosmologist will be alternately delighted and dismayed to see many half-forgotten theories receiving an airing. From the Lyttleton-Bondi cosmology to modern gauge theories of gravity, Wesson gets around to them all in some context or other.

Like it or not, many of these unusual theories have been extensively, even infuriatingly, discussed in the scientific literature for years. Some of them, such as

the so-called big number coincidences or the varying G theories, have occupied the attentions of eminent physicists and astronomers. One should not blame the author for attempting a comprehensive presentation of such issues just because they command only minority support.

My real criticism of this book is that it reads rather like a shopping list. In his enthusiasm for including everything, the author has only managed to achieve a compendium of speculations, and not a coherent discussion. References are so liberally used that the text becomes impossible to read in some parts. On one page I counted one hundred such interjections. This is fine in a reference source for non-standard cosmology and astrophysics, but will not satisfy the demand by conservative "straight thinkers" to know something of the work of their more adventurous-minded colleagues.

Of course, a subject does not become

respectable merely by quoting a vast array of big names. Even Einstein patronized some odd theories. Nor should one be persuaded that all unconventional ideas are in some mysterious way consistent with each other. This book is written in a style that leads one to believe there is a sort of grand, coherent theory of unconventional physics and cosmology existing alongside the more publicized one. It gives the impression of two sciences: the one to which most professionals subscribe, and another, almost the same in its predictions, but with subtle differences. Wesson is fair in drawing attention to some of the shortcomings of the latter, but clearly believes that his several hundred "grey area" references are probing closer to the truth than many of us would be prepared to acknowledge. □

Paul Davies is Professor of Theoretical Physics at the University of Newcastle upon Tyne.

Agricultural prognoses

Kenneth Blaxter

Britain's Future in Farming. Edited by Sir Frank Engledow and Leonard Amey. Pp.164. (Geographical Publications: Berkhamsted, Herts, UK, 1980.) £9.

THE farming industry accounts for less than 3% of the labour force in the United Kingdom and contributes about the same percentage to its gross domestic product. Yet it is the most obvious of our industries since it occupies the major part of our countryside and produces most of the food we consume. The industry has much changed with time; it is now capital intensive and heavily dependent in some of its sectors on sophisticated inputs from other parts of the economy. These changes are relatively recent and, as the authors of this book state, "a highly urbanized economy with a romantic view of the countryside has yet to come to terms with the unpicturesque aspects of an intensive and efficient agriculture". Questions necessarily arise about the future of farming, in turn inevitably implying questions about the policy to be adopted and the goals to be sought; these considerations are basic to this volume.

The book is the result of four years of study by a group of 15 people knowledgeable about farming, food provision, forestry, agricultural science, banking, and the social and political history of land use. None of the chapters is assigned to an author, but the discerning can detect where initial responsibility rested for written styles are revealing. There is thus a collective responsibility and this at times seems to have engendered some collective irresponsibility. Surely

some of the statements made cannot have been agreed by all. Is it reasonable to suppose that all agreed "that the Ministry of Agriculture [MAFF] should resume its role in education and research and subsume the Agricultural Research Council [ARC]"? Or that security of tenure for farmers should be withdrawn forthwith? Such statements in the text do not appear in the conclusions with which no doubt all agreed. The first becomes transformed to a plea for better integration and a suggestion that attention should be drawn to the relationship of MAFF and ARC, while the second does not appear at all.

A few provocative statements, however, do not detract from the overall thesis and, on the contrary, perhaps enhance what is a sensible account of the problems related to the management of our resources of land. It should do much to show that the interests of agricultural and urban communities in reality have much in common and that certain broad principles related to land use may well be sufficient to provide a basis on which to develop policies to meet new eventualities. The conclusions and recommendations are admittedly detailed — there are 28 of them — but the whole tenor of the book relates to the theme that the source of renewable land must be preserved, that the pressures upon it whether direct or indirect must be reduced and farming practices themselves should be designed to provide a flexibility to accommodate inevitable and continued change. □

Sir Kenneth Blaxter is Director of the Rowett Research Institute, Aberdeen.

Practical vacua

John Yarwood

A User's Guide to Vacuum Technology. By John F. O'Hanlon. Pp.402. (Wiley-Interscience: 1980.) £13.35, \$31.20.

JOHN O'HANLON is a member of the research staff at IBM in the USA. As a result this book is really up-to-date. He has written the most useful and thoughtful account of vacuum technology which has appeared in recent years, and one which will be of value to the student, technician, engineer, scientist and technical manager. As would be expected from an author with such a background, the book (according to his preface) emphasizes "the operation and selection of equipment for processes used in semiconductor, optics, and related technologies". But he does himself an injustice — this book is much wider in scope than such a statement would appear to indicate.

SI units are used throughout, with the exception that pumping speed (now called "volume rate of flow" — not a good term although scientifically accurate) is given in l/s or m³/h, depending on the type of pump, and the diameters of the top flanges of diffusion pumps are given in inches. The section on essential gas theory covers gas properties, kinetic theory, basic relevant formulae (without proofs), gas flow and the measurement of pumping speed (where the work of the International Standards Organization has not been acknowledged; all the credit seems to go to the USA). Succeeding chapters are up-to-date, apposite accounts of real value, dealing with a wide range of equipment — gauges, pumps, analysers — and associated topics.

The book is rounded off with six appendices and, overall, can be recommended as a most useful and comprehensive volume.

John Yarwood was formerly Head of Physics at the Polytechnic of Central London, and is the editor of Vacuum.

UV photobiology

Philip C. Hanawalt

Biological Effects of Ultraviolet Radiation. By Walter Harm. Pp.216. (Cambridge University Press: 1980.) Hbk £15, \$29.95; pbk £4.95, \$9.95.

WE HAVE long appreciated the essential role that light plays in the maintenance of life forms on our planet through the processes of photosynthesis. In recent years, however, we have become increasingly aware of the deleterious effects of the shorter wavelengths of light in the Sun's spectrum. We have learned that ultraviolet

light (UV) damages the precious DNA, resulting in mutations, the death of cells and sometimes malignant transformation. The latter effect would be intolerably frequent were it not for cellular enzymes that constantly monitor the DNA to identify and repair the lesions that UV inflicts. This is dramatically illustrated by the rare hereditary disease, xeroderma pigmentosum, in which DNA repair deficiency renders the victims exquisitely sensitive to sunlight-induced skin tumours.

Biological effects of UV was an excellent choice of topic for this first volume in a new series sponsored by the International Union of Pure and Applied Biophysics, since UV is both an important biophysical probe for DNA repair systems and an environmental hazard of some concern. The books in this series are meant to serve as specialized texts for advanced students in particular fields of biophysics. The author, Walter Harm, was one of the pioneers in the field of UV photobiology and he was therefore in a good position to provide the kind of historical perspective that could bring the subject to life and excite students. Unfortunately this monograph doesn't really gain much vitality as it plods ponderously through the details of classical bacterial UV photobiology.

Without first surveying the range of interesting biological effects, the first chapter launches into electronic excitation and the practical nuts-and-bolts details of experimental UV photobiology. Chapter 2 carries on with more practical advice for the researcher, including some useful numerical examples to illustrate important points. A carefully detailed consideration of UV interactions with biologically important molecules is followed by a not-so-useful chapter on classical target theory and survival curve shapes. (The word "multihitlike" was a new one to me.) I would have preferred more discussion of why shoulders on survival curves may be explained by existence of repair systems. The terms excision-repair and post-replication repair are introduced but not defined until three chapters later.

The subject of enzymatic photo-reactivation is treated in great detail, reflecting the recent research interests of the author. In contrast, the discussion of dark repair systems is incomplete and contains a number of inaccuracies, such as a reference to sequential action of the *uvrA* and *recA* systems (p.107). The book is nearly devoid of discussion of our current understanding of the enzymology of dark repair and its genetic control. Yet this is perhaps one of the most exciting areas of research in UV photobiology.

The predominant emphasis in the text is on UV inactivation of bacteria and their phage, even in the chapter on solar UV radiation effects. Sunlight effects on human skin are relegated to less than two pages followed by a brief chapter on UV carcinogenesis and an even shorter one on sensitized UV effects on DNA.

I must say that I would find it difficult to teach a course using this book as a text but I find it quite useful as a reference work. It does provide a comprehensive treatment of the now classical studies on UV effects in prokaryotes. It might have been more aptly titled *Microbial Effects of UV Radiation*; as Dr Harm emphasizes, much of our

understanding in UV photobiology and DNA repair has been based upon the solid foundation of early studies with simple prokaryotes. □

Philip C. Hanawalt is Professor of Biology and Dermatology at Stanford University, and Director of the Graduate Program in Biophysics.

Social science and the stomach

John Yudkin

Consuming Passions: The Anthropology of Eating. By Peter Farb and George Armelagos. Pp.279. (Houghton Mifflin: 1980.) \$12.95.

THE past 25 years have seen not only a rapid growth in research in nutrition, but the increasing recognition that nutrition is as much a social as an experimental science. Its wide span is encompassed in the short definition of nutrition as the study of the relationship between people and their food. Thus, there are now fewer academics who consider nutrition to be a section of biochemistry or of physiology, and few among the growing number of university departments of nutrition that do not include in their courses relevant aspects of social nutrition from economics, sociology, anthropology and psychology.

This book will certainly be of interest to nutritionists and dietitians, as well as to anthropologists, but its appeal is very much more general since it contains a mass of fascinating information. Its wide range is best indicated from this quotation near the end of the work: "The thesis of this book has been that to know what, where, how, when and with whom people eat is to know the character of their society". For example, you will learn from the book that "cannibal" is derived from "Carib" (as in "Caribbean") and that a "companion" is the person with whom you eat bread; that among the people of Trobriand it is unseemly for couples to eat together before marriage, just as it is (was?) for unmarried Western couples to sleep together; that cultural customs such as not eating pork or the reverence of the Hindu for cattle often have a biological or ecological basis.

The authors deal first with the evolution of human dietary patterns, how they have adapted to the availability of local foods, and the differences called for, and met, at different phases of the life of the individual. There follows a description and suggested explanation for the myths and religious concepts that influence eating behaviour, and the rules that different people have adopted towards food in their relationships with family, clan and strangers. There is considerable discussion of food preferences and avoidances, and the development of national cuisines.

It would be pleasing to be able to say that this book had achieved a synthesis between those aspects of eating that belong to the social sciences and those that belong to the natural sciences. The fact is, however, that until recently at any rate most people who have become professionally interested in nutrition have come through the separated and narrowly confined and demarcated pathways of these two groups of disciplines. It is understandable then — but a great pity — that the data on the nutrient content of foods have not been properly checked.

It is not true that one person may "burn calories ten times faster" than another "of the same age, sex and physical proportions". It is misleading, too, to worry about the fact that a portion of canned peas contains 100 times as much salt as do fresh peas, when in absolute terms this would still be about one-fiftieth of the amount taken in a day; more importantly, it ignores the fact that people add very much more salt when cooking vegetables than that present in the canned peas. The plausible suggestion that pica usually has a nutritional significance — for example, that eating clay provides minerals lacking in the diet — has been repeatedly disproven.

One last grumble is that the authors have adopted the somewhat hysterical current concern about the safety of modern food processing. For example, they say categorically that food colours are carcinogenic, a statement that, of all countries, is highly unlikely to be true in America with its rigid food laws that are especially sensitive about suspected carcinogenicity of food additives.

Nevertheless, for the general reader this is a book that is well and clearly written (except for the use of "human" as a noun!) and is full of interesting facts about what and why we eat what we do. For the specialist reader, too, there is a host of references. If, therefore, you are dubious about the theories of Claude Levi-Strauss on attitudes and behaviour towards food, you will be directed to the writings of several other authors, some of whom agree and some of whom disagree with his views. □

John Yudkin is Emeritus Professor of Nutrition at London University.

CORRESPONDENCE

Continued from page 218

provisions of the First Amendment of the Constitution by permitting the teaching of evolution. This, the suit says, disparages the religion of creationists and in itself amounts to the teaching of a religion — the religion of secular humanism.

The charges in the suit focus on the *Science Framework for California Public Schools, Kindergarten and Grades One through Twelve*, published by the California State Department of Education in 1978. Dr Richard Lemmon and I served on the committee that wrote this publication, a task that took about three years. It involved a steady battle with creationists who resisted the inclusion of statements concerning evolution. On some occasions, we obtained their consent, only to have them change their minds. For example, a statement that "Darwinian evolution is a cornerstone of modern biology" was first permitted, then deleted.

The final document says:

Living organisms have universal properties, including derivation of energy from outside sources, and reproduction. Evolutionary studies indicate that these organisms are naturally selected from generation to generation, producing descendants with different characteristics and producing variability among populations of living species. The process has been going on so long that it has produced all the groups and kinds of plants and animals now living as well as others that have become extinct.

... In addition to reproduction, another characteristic of life is change in its genetic material with passage of time. This process, termed evolution, takes place through changes in DNA. Changes in DNA molecules are produced by mutation, which includes replacement of some DNA bases by others, and recombination in which segments of DNA are added to or subtracted from genes. Duplication of genes sometimes also occurs. Most mutations are harmful and do not persist; they are eliminated by natural selection.

Beneficial mutations occasionally take place and are responsible for the appearance of new characteristics. A third class is "neutral" or "near neutral" mutations.

The Creation Research Society (through Creation-Life Publishers) does not confine itself to matters of religion but also addresses scientific questions. A complaint by a parent in Livermore is that the publication *Dry Bones*, published by Creation-Life in 1979, is being used in lower grades of an elementary school. It contains a dialogue between children and their father, including:

Q: "Did dinosaurs live when Noah lived, Dad?"

A: "That's what I think. The way it looks, dinosaurs were drowned in the Flood, too."

Q: "Did Noah have to take them on the Ark?"

A: "He surely did". . .

Q: "I guess [Darwin] thought fossils were proof for evolution."

A: "No, Darwin said that fossils were 'perhaps the most obvious and serious objection' to his theory."

A: "... Did your friend also tell you about

scientists at Oak Ridge National Laboratories?"

Q: "No, what about them, Dad?"

A: "They used uranium dating on wood in rocks of the dinosaur group, and got ages of only thousands of years."

THOMAS H. JUKES

University of California, Berkeley

Genes and race

SIR — Some time ago you published a letter from me concerning the use made by the National Front of apparently "scientific" justification of racist arguments. I asked, through your columns, Professors Eysenck and Jensen publicly to dissociate themselves from the use made of their names in apparent support of these views.

I now wish to bring an analogous issue to the attention of readers of *Nature*. Ever since the publication in 1975 of E.O. Wilson's *Sociobiology: The New Synthesis*, the danger has been clear that in due course racists would deploy sociobiology in support of their views. The "New Right" in France has been doing so for some time, and now the breakaway journal from the National Front in Britain, *New Nation*, has done so too. Its first two issues, dated summer and autumn 1980, carry articles entitled: "Nationalism, racialism: products of our selfish genes" and "Science is championing our creed of Social Nationalism", authors respectively John Thornton Bannerman and Richard Verrall (the latter is also the author of a pamphlet entitled "Did Six Million Really Die?").

The first cited of these comes complete with a picture of "Richard Dawkins, author of *The Selfish Gene*", and cites him, Wilson, Maynard Smith and one Trivers (presumably Trivers) amongst others. The final section of this article appears alongside an advertisement from "Nationalist Books" which includes such choice titles as *Debunking the Genocide Myth* and *Anne Franck's diary — a hoax*. Opponents of sociobiology, it is therefore scarcely surprising to note, are categorized as "Marxist" and "Jewish".

The concluding paragraph reads as follows:

For us, as racial nationalists, this is an important vindication of our position. For it is increasingly clear that it isn't just "bad luck" that our genes don't permit us to live in a Marxist-Rousseauesque egalitarian communist utopian World State of universal altruism. It was an inevitable result of the way evolution works that our genes would not permit us so to live. What the evolutionary theoreticians have shown us is that, with the system of genetic inheritance shared by all vertebrates, the only type of social organization which can evolve, let alone work, is one based upon kinship, upon the ties of blood and of race. Nationalism is not only an integral part of our genetic inheritance, it is an inevitable end product of the evolutionary processes which shaped that inheritance.

May I suggest that it would be in the public interest that John Maynard Smith and Richard Dawkins should clearly dissociate themselves from the use of their names in support of this neo-Nazi balderdash.

STEVEN ROSE

The Open University,
Milton Keynes, UK

Energetic consensus

SIR — It was disheartening to learn from your correspondent that the First European Bioenergetics Conference had shown a "general acceptance of the chemiosmotic concept of energy transduction" and that interest was now moving on from this assured base to the finer molecular details of the problem (*Nature* 4 December 1980, p.432). If there is such consensus on this matter there is very good reason to believe that it could be mistaken.

The chemiosmotic hypothesis is founded upon a very simple and invalidating illogicality: no evidence has ever been given that protons move into the bulk-phase during synthesis. Oxygen-pulse studies which supposedly support the hypothesis are carried out under entirely different experimental circumstances; and to have used the results of such studies to generalize upon the nature of proton movements in ATP synthesis was to introduce an inferential *non sequitur* into the energy transduction argument from the outset. The basic chemiosmotic equation:

$$\Delta p = \Delta \psi - Z \Delta pH \quad (1)$$

has no meaning if protons are not translocated to the bulk-phase.

We have attempted to state this view since 1974¹ and the accumulating experimental evidence we have obtained has led to the *q*-zone interpretation of mitochondrial energy transduction in which the proton-motive force has been regarded as a localized and surface electrical force, with a minimal osmotic involvement:

$$\Delta p^e \approx \Delta \psi^e \quad (2)$$

Recent experiments showing the generation of negative fixed charges on the inner membrane specifically associated with the energization process² have fulfilled the main prediction of the *q*-zone interpretation and given a further compelling reason to suspect the adequacy of the chemiosmotic view; they also call into question our own formulation of the proton-motive force given in equation (2).

It now seems probable that during synthesis the proton-motive force should be regarded as a coulombic rather than a chemiosmotic force, and that its assessment should be made in terms of charge-charge relationships taking place in a surface region of variable dielectric. Some of the more recondite thermodynamic implications of such a system have already been anticipated by Ashcroft and Coster³; meanwhile an unsophisticated estimate of the interchange distance, *l*, may be gained from the relationship:

$$W = \frac{q_1 q_2}{\epsilon l} = 15 \text{ kcal } (\Delta G_p)$$

and the speculative use of a now dubious chemiosmotic H^+ / ATP stoichiometry of 3. Substituting the dielectric constants for the "limit" media ($\epsilon_{aq} = 75$; $\epsilon_{lipid} = 2$) interchange distances of 0.9 and 33 Å are obtained; the functional value may reasonably be expected to lie between these extremes and to be in accord with the requirements of the *q*-zone interpretation.

F.H. MALPRESS

Department of Biochemistry,
The Queen's University of Belfast, UK

1. Archbold, G.P.R. *et al.* *Biochem. Soc. Trans.* 2, 751-754 (1974).
2. Archbold, G.P.R. *et al.* *Biochem. Internat.* 1, (in the press).
3. Ashcroft, R.G. & Coster, H.G.L. *Bioelectrochem. Bioenerg.* 5, 37-42 (1978).

ANNOUNCEMENTS

Awards

Drs J.-P. Mach and S. Carrel, (Lausanne Branch of the Ludwig Institute for Cancer Research) have received the 1980 Cancer Prize of the Swiss Cancer League.

The Charles Frederick Chandler Medal, presented by Columbia University for achievement in pure or applied chemistry has been awarded to **Frank H. Westheimer** (Morris Loeb Professor of Chemistry at Harvard University).

The NASA Group Achievement Award has been presented to **Dr Sam Gulkis** (JPL); **Dr David Jauncey** and **Dr Michael Batty** (CSIRO) for the development of a radio astrometric interferometer in the Southern Hemisphere.

The Council of The Institute of Physics has made the following awards for 1981; Bragg Medal and Prize to **G.E. Foxcroft** (Rugby School); Charles Chree Medal and Prize to **Dr K.A. Browning** (Meteorological Office Radar Research Laboratory, RSRE Malvern); Charles Vernon Boys Prize to **Dr P.N. Pusey** (Royal Signals and Radar Establishment, Malvern); Duddell Medal and Prize to **Dr B.A. Joyce** (Philips Research Laboratories, Redhill); Glazebrook Medal and Prize to **Dr G.H. Stafford** (Rutherford and Appleton Laboratories, Chilton); Guthrie Medal and Prize to **Dr J.C. Ward** (Physics Department, Macquarie University, Australia); Maxwell Medal and Prize to **Dr J.M. Kosterlitz** (Department of Mathematical Physics, Birmingham University); Thomas Young Medal and Prize to **N.J. Phillips** (Department of Physics, Loughborough University of Technology); Prize for the 1980 Graduateship Examination of the Institute to **James Cooke** (Dublin College of Technology).

The Council of the Royal Society invite nominations for the 1981 Royal Society Esso Energy Award which should be sent by 6 February 1981. The Award is given for outstanding contributions to the advance of science or engineering or technology leading to the more efficient mobilization, use and conservation of energy resources, and consists of a gold medal and a prize of £1,000. Further information from: **Dr R.W.J. Keay**, The Royal Society, 6 Carlton House Terrace, London SW1, UK.

A fellowship in human anatomy, to be called the Wellcome-Franks Medical Fellowship, has been endowed at St Hugh's College, Oxford, by The Wellcome Foundation Limited. Enquiries to: **Ron Champion**, The Wellcome Foundation Ltd, 183 Euston Rd, London NW1, UK.

Environmental researchers and scientists — either from university or industry — are invited to compete for Chemviron's fifth 'Award' a \$10,000 cash prize to be given in 1982. It is awarded for outstanding work in the area of physical-chemical treatment of potable and waste water, or of sludge resulting from treatment of such waters. Copies of the regulations can be obtained from **Dr J. Melbourne**, c/o Chemviron, Chaussee de Waterloo 1135, B-1180, Brussels, Belgium. Only entries originating in Europe will be accepted.

The Roussel Prize to be awarded in 1982, is given every two years to a chemist or a biochemist whose work on steroids in therapeutic medicine has been chosen as the most outstanding by an International Committee. Further information from: **Professor J. Mathieu**, Centre de Recherches, Roussel Uclaf, 102 route de Noisy, 93230 Romainville, France.

The Trustees of the Lady Tata Memorial Trust invite applications for awards for personal support for research on leukaemia, in the Academic Year beginning October, 1981. Candidates with programmes of research on any aspect of malignant disease which may throw light on problems of leukaemia will be eligible for consideration. Further information from: Secretary of the (European) Scientific Advisory Committee, Lady Tata Memorial Trust, MRC Leukaemia Unit, Royal Postgraduate Medical School, Du Cane Road, London W12, UK.

The Paul-Martini-Prize, was initiated by the Medizinisch Pharmazeutische Studiengesellschaft e.V. (Mainz) in association with the Deutsche Gesellschaft Für Medizinische Dokumentation, Informatik und Statistik e.V. to promote the development of scientific methods for evaluation in clinical pharmacological and therapeutical fields. Papers should be submitted by 28 April 1981 either in German or English with six copies to the Medizinisch Pharmazeutische Studiengesellschaft e.V. Bilhildstr. 2, D 6500 Mainz.

The International Union Against Cancer, with funds provided by the Cancer Research Campaign (UK), will award fellowships for research on cancer to persons on the staff of universities, teaching hospitals, research laboratories or similar institutions. These enable investigators to work abroad to gain new experience in clinical or basic research laboratories or similar institutions. Application forms and additional information from: International Union Against Cancer, rue du Conseil-General, 3, 1205 Geneva, Switzerland.

Meetings

9-10 February, **New Concepts in Drug and Nutrition Delivery Systems**, Paris (Roberts S. First, Inc. Avenue Marnix 19A, 1050 Brussels, Belgium).

9-13 February **Fundamentals of Microprocessors**, London (Bleasdale Computer Systems Ltd, Francis House, Francis St, London SW1, UK).

9-13 February, **Protein Structure and Function**, Lorne, Australia (Dr R.J. Simpson, St Vincent's School of Medical Research, Victoria Parade, Fitzroy, Victoria, Australia).

12 February, **4th Interdisciplinary Cancer Research Workshop**, New Orleans (Dr P. Politzer, Chemistry Dept, University of New Orleans, Louisiana).

15-17 February, **Proprioception, Posture and Emotion**, New South Wales (Dr D. Garlick, Committee in Postgraduate Education, PO Box 1, Kensington, New South Wales, Australia 2033).

8-15 February, **Immunoglobulin Idiotypes and their Expression**, Salt Lake City (ICN-UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

15-20 February, **Differentiation and Function of Hemopoietic Cell Surfaces**, Keystone (ICN-UCLA, Molecular Biology Institute, University of California, Los Angeles, California 90024).

20 February, **Ulcer Therapy**, Frankfurt (GDCh, Postfach 90440, D-6000 Frankfurt/M8, West Germany).

25-27 February, **Recombinant DNA Research**, San Francisco (Edward Ruffig, c/o Scherago Associates Inc, 1515 Broadway, New York 10036).

22 February — 1 March, **Mechanisms of Chemical Carcinogenesis**, Keystone (ICN-UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

1-8 March **Cellular Recognition**, Keystone (ICN-UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

4-7 March, **Chemical Indices and Mechanisms of Organ-directed Toxicology**, Barcelona (Secretariat, Toxicology 81, 142 Oxford Rd, Cowley, Oxford, UK).

8-21 March, **Basic Concepts and Techniques in Research on Biological Membranes**, Ibadan (Dr Enitan A. Bababunmi, Dept of Biochemistry, University of Ibadan, Nigeria).

9-11 March, **Drug Delivery System Development**, London (A.S. Goldberg, Powder Advisory Centre, PO Box 78 London NW11, UK).

10-13 March, **POWTECH '81**, Birmingham (Clive H.R. Hopkins, Green Dragon House, 64-70 High St, Croydon, Surrey, UK).